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(54) Title: CHEMICAL PROBES FOR LEFT-HANDED DNA AND CHIRAL METAL COMPLEXES AS Z-SPECIFIC ANTITUMOR AGENTS
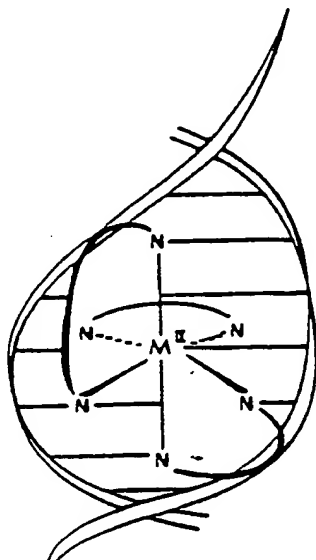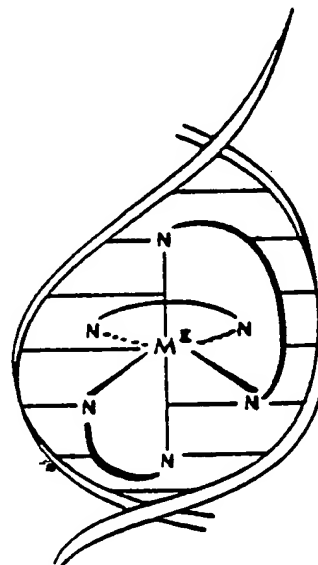
Lambda   Delta

(57) **Abstract**

A coordination complex and salts and optically resolved enantiomers thereof, of the formula $(R)_3$---M, wherein R comprises 1,10-phenanthroline or a substituted derivative thereof, M comprises a suitable transition metal, e.g. ruthenium(II) or cobalt(III), and R is bonded to M by a coordination bond. The complexes of this invention are useful in methods for labeling, nicking and cleaving DNA. The lambda enantiomer of complexes of this invention is useful in methods for specifically labeling, detecting, nicking and cleaving Z-DNA. The complexes may also be used in a method for killing tumor cells and may be combined with a pharmaceutically acceptable carrier to form a pharmaceutical composition for the treatment of tumor cells in a subject. The invention further concerns methods for treating a subject afflicted with tumor cells.

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CHEMICAL PROBES FOR LEFT-HANDED DNA AND
CHIRAL METAL COMPLEXES AS Z-SPECIFIC ANTITUMOR AGENTS

10

Background of the Invention

Much of the information set forth herein has been pub-
lished. See Barton, J.K. et al., J. Am. Chem. Soc.,
15 1984, 106: 2172-2176 (April 6, 1984); Barton, J.K. and
Raphael, A., J. Am. Chem. Soc., 1984, 106: 2466-2468
(April 18, 1984); Barton, J.K. et al., Proc. Natl. Acad.
Sci. USA, 1984, 81:1961-1965 (April 27, 1984); and
Barton, J.K., J. Biom. Structure and Dynam., 1983, 1:
20 621-632 (January 18, 1984). The above-mentioned papers
were distributed by the respective publishers on the
dates provided in parentheses.

Throughout this application various publications are
25 referenced by arabic numerals within parentheses. Full
citations for these publications may be found at the end
of the specification immediately preceding the claims.
The disclosures of these publications in their entire-
ties are hereby incorporated by reference into this
30 application in order to more fully describe the state
of the art to which this invention pertains.

The binding of heterocyclic compounds to DNA by inter-
calation, where the planar aromatic cation stacks be-
35 tween adjacent base pairs of the duplex (1), has been
the subject of considerable investigation (2-4).

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Intercalative drugs can be strongly mutagenic, and some, as adriamycin and daunomycin, serve as potent chemotherapeutic agents (5). The small intercalators such as ethidium or proflavine in addition provide useful chemical probes of nucleic acid structure (6). Metallointercalators have been particularly useful in probing DNA structure and the intercalation process itself, because the ligands or metal may be varied in an easily controlled manner to facilitate the individual application (7).

The aromatic chromophore of the intercalative cation can provide a sensitive handle to monitor the conformation and flexibility of the helix. Many intercalators show antibacterial or anticancer activity and, because the inserted residue often resembles a base pair in shape and thickness, intercalators are commonly frame shift mutagens. (3) Intercalation appears to require, simply, a planar heterocyclic residue, (4) and in fact cationic metal complexes which contain aromatic ligands bind to DNA by intercalation as well. (5) Platinum intercalators have uniquely provided electron dense probes for x-ray diffraction experiments. (6) Moreover the metallointercalation reagents have offered particular experimental flexibility in that both the metal and accessory ligands may be varied for individual applications. Comparisons of the binding of the intercalative 2,2'-bipyridylplatinum(II) reagent with the analogous nonintercalating bis(pyridine)platinum(II) species by fiber X-ray diffraction methods, for example, demonstrated quite simply the requirement for ligand planarity in the intercalation process (8).

The original studies of metallointercalators centered on square-planar platinum(II) complexes containing aromat-

The original studies of metallointercalators centered on
square-planar platinum(II) complexes containing aromat-
ic terpyridyl or phenanthroline ligands (9), and sin-
gle-crystal studies of terpyridylplatinum(II) species
stacked with nucleotides showed the platinum complex to
insert almost fully between the base pairs (10,11).
More recently the reagent methidiumpropyl-Fe(II)EDTA,
which contains a redox-active metal center tethered to
an organic intercalator, has been applied in "foot-
printing" experiments to determine the sequence spec-
ificities of small drugs bound to DNA (12). Bis(phen-
anthroline)-cuprous ion (13) has similarly been em-
ployed in DNA cleavage experiments (14), and this rea-
gent also presumably binds initially to the DNA by
intercalation.   (3,5,6,8-tetramethyl-1,10-phenanthro-
line)$_3$Ru(II) has been reported and its use against
influenza virus, fungus, yeast and leukemia investigated
(98). The ability of the tris tetramethyl complex to
bind to or cleave DNA has not been reported. Further-
more, enantiomers of that complex have not been sepa-
rated and their respective properties compared.

Reagents of high specificity and even stereoselectivity
would be desirable in the design both of potent drugs
and of structural probes. For the chiral complex
(phen)$_3$Zn^{2+} (phen = 1,10-phenanthroline) an enantio-
meric preference in binding to B-DNA has been observed
(15). As for the tetrahedral (phen)$_2$Cu$^+$ complex, and in
contrast to the square-planar platinum intercalators,
the octahedral coordination in the tris(phenanthroline)
metal cations can permit a partial insertion of only one
coordinated ligand. Thus while one ligand is stacked
between base pairs, the remaining nonintercalated phe-
nanthroline ligands should be available to direct the
enantiomeric selection.

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The left-handed DNA helix has received considerable
attention since the original crystallographic study of
the Z-DNA fragment $[d(CpG)]_6$ (16). Solution conditions
that include high ionic strength (17), hydrophobic sol-
5   vents (18), the presence of certain trivalent cations
(19), or covalent modification with bulky alkylating
agents (19-23) all facilitate the transition of a right-
handed double helix into a left-handed form. This
striking conformational transition was first observed
10 for poly[d(G-C)] (17). More recently, the alternating
purine-pyrimidine sequence $[d(G-T)]_n \cdot [d(C-A]_n$ has been
shown to form Z-helices as well (24, 25). Methylation
of cytosine residues at carbon-5 lends stability to Z-
form DNA (19, 26) and, under physiological conditions,
15 transitions to a left-handed structure can occur to
relieve the torsional strain in underwound negatively
supercoiled DNA (27-29). These latter findings suggest
mechanisms for left-handed DNA formation in the cell,
where such structures could be important in controlling
20 gene expression. Negatively supercoiled simian virus 40
DNA, for example, has been found to contain potentially
Z-DNA-forming alternate purine-pyrimidine regions with-
in transcriptional enhancer sequences (30).

25 To explore any biological role for left-handed DNA,
sensitive and selective probes are required. Assays of
superhelix unwinding, NMR experiments and circular
dichroism have been used in detecting Z-DNA. These
methods, however, are indirect, do not assay for helix
30 handedness, and require large quantitites of material.
More recently antibodies to Z-DNA have been elicited.
The antibodies provide a more sensitive means of detec-
tion. Z-DNA appears to be a strong immunogen. Anti-Z-
DNA antibodies have been elicited with both brominated

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poly[d(G-C)] (31) and poly[d(G-C)] modified with diethy-
lenetriamineplatinum(II) (32) as antigens. The struc-
tures of Z-DNA and in particular of a modified Z-form
provide a multitude of antigenic characteristics: the
5   left-handed helicity, the zigzag dinucleotide phosphate
repeat, the protruding purine substituents in the shal-
low major groove. It is not surprising then that the
various antibodies obtained appear specific for differ-
ent localized features of Z-DNA (33). The development
10 of a specific chemical probe, so designed as to recog-
nize a known structural element of Z-DNA, e.g. the helix
handedness, would offer a simple complementary approach
but has not heretofore been reported.

15 Enantiomeric selectivity has been observed in the in-
teractions of tris(phenanthroline) metal complexes with
B-DNA (15, 34-35). Experiments with tris(phenanthro-
line)zinc(II) have indicated stereoselectivity (15);
dialysis of B-DNA against the racemic mixture leads to
20 the optical enrichment in the Λ enantiomer. Subsequent
luminescence, electrophoretic, and equilibrium dialysis
studies of the well-characterized ruthenium(II) ana-
logues have shown that the tris(phenanthroline) metal
isomers bind to DNA by intercalation and it is
25 the Δ enantiomer that binds preferentially to a right-
handed duplex (34,35). The enantiomeric selectivity is
based on steric interactions between the nonintercalated
phenanthroline ligands and the phosphate backbone.
Although the right-handed propeller-like isomer interca-
30 lates with facility into a right-handed helix, steric
repulsions interfere with a similar intercalation of
the Λ enantiomer.

35 Based on this premise, tris(phenanthroline) metal com-
plexes appear useful in the design of probes to distin-

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guish left-handed and right-handed DNA duplexes. The design flexibility inherent in metallointercalation reagents, in which both ligand and metal may be varied easily, makes the coordination complexes attractive probes (7,8,35). We have concentrated here on phen-anthroline complexes of ruthenium(II) because of the high luminescence associated with their intense met-al-to-ligand charge-transfer band (37, 38) and because the exchange-inert character of the low-spin d^6 complexes limits racemization (20).

Furthermore, there has been considerable interest in DNA endonucleolytic cleavage reactions that are activated by metal ions, (39,40) both for the preparation of "footprinting" reagents (41) and as models for the reac-tivity of some antitumor antibiotics, notably bleomycin (42) and streptonigrin. (43) The features common to these complexes are that the molecule has a high affin-ity for double-stranded DNA and that the molecule binds a redox-active metal ion cofactor. The delivery of high concentrations of metal ion to the helix, in locally generating oxygen or hydroxide radicals, yields an efficient DNA cleavage reaction. Additionally, co-balt(III) bleomycin cleaves DNA in the presence of light.(44)

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Summary of the Invention

This invention concerns a coordination complex or salt thereof having the formula $(R)_3$---Co(III), wherein R comprises 1,10-phenanthroline or a substituted derivative thereof and R is bound to Co by a coordination bond.

One embodiment concerns a method for labeling DNA with a complex of the formula $(R)_3$---M, wherein R comprises 1,10-phenanthroline or a substituted derivative thereof, M comprises a suitable transition metal, and R is bonded to M by a coordination bond. In this and other embodiments a suitable transition metal is one which is capable of forming an octahedral complex with R, such as ruthenium (II) or cobalt (III). The invention also concerns a DNA molecule labeled with a complex of the formula $(R)_3$---M, as defined above, wherein the complex is bound to the DNA by intercalation.

Another embodiment of this invention is a method for selectively labeling Z-DNA with the lambda enantiomer of a complex of the formula $(R)_3$---M, as defined above. This method comprises contacting the lambda enantiomer of the complex under suitable conditions such that the complex binds to the Z-DNA. The invention further involves a labeled DNA molecule comprising Z-DNA and the lambda enantiomer of a complex of the formula $(R)_3$---M, as defined above, the complex being bound to the Z-DNA.

Another embodiment of this invention concerns a method for detecting the presence of Z-DNA. This method involves selectively labeling Z-DNA according to the above-mentioned method and detecting the presence of the complex bound to the Z-DNA.

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Still another embodiment of this invention is a method
for nicking double-stranded DNA by effecting breakage of
at least one phosphodiester bond along the DNA. The
method involves contacting the DNA with a cobalt (III)-

5   containing complex of this invention under suitable
conditions such that the complex intercalates into the
DNA to form an adduct and irradiating the adduct so
formed with a sufficient dose of ultraviolet radiation
of an appropriate wavelength so as to nick the DNA at

10   the site(s) of intercalation. This invention further
involves a method for cleaving double-stranded DNA which
comprises nicking the DNA by the above-mentioned method
and treating the nicked DNA so produced with a suitable
enzyme capable of cleaving single-stranded DNA under

15   effective conditions to cleave the nicked DNA at the
site of the nick(s).

An additional embodiment of this invention is a method
for selectively nicking Z-DNA by effecting breakage of

20   at least one phosphodiester bond along the Z-DNA. The
method involves contacting a DNA molecule containing a
Z-DNA sequence with a lambda enantiomer of a cobalt
(III)-containing complex of this invention under suita-
ble conditions such that the complex binds to the Z-DNA

25   to form an adduct and irradiating the adduct so formed
with a sufficient dose of ultraviolet radiation of an
appropriate wavelength so as to nick the DNA at the
binding site(s). Double-stranded Z-DNA may be selec-
tively cleaved by selectively nicking the Z-DNA by the

30   above-mentioned method and treating the nicked DNA so
produced with a suitable enzyme capable of cleaving
single-stranded DNA under effective conditions to cleave
the nicked double-stranded DNA at the site of the
nick(s).

35

This invention further concerns a method for killing a portion of a population of appropriate tumor cells. The method involves contacting the tumor cells under suitable conditions with an effective amount of the lambda enantiomer of a coordination complex of the formula $(R)^3$--M, as previously defined, to kill the tumor cells. In another embodiment a racemic cobalt(III)-containing complex of the invention may similarly be used to kill tumor cells. When a cobalt (III)-containing complex is used, the method may further comprise irradiating the tumor cells with a suitable dose of ultraviolet radiation of an appropriate wavelength at a suitable time after the tumor cells have been contacted with the complex, permitting the complex to nick DNA.

Still another embodiment of this invention is a pharmaceutical composition for the treatment of tumor cells in a subject. The pharmaceutical composition comprises a pharmaceutically acceptable carrier and an effective anti-tumor amount of a cobalt(III)-containing complex of the invention or of the lambda enantiomer of a complex of the formula $(R)_3$---M, as defined previously. Such a composition may be used in a method for treating a subject afflicted with tumor cells so as to cause regression of the tumor cells. This method involves administering to the subject by a suitable route the pharmaceutical composition in an amount sufficient to cause regression of the tumor cells.

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Brief Description of the Figures

Fig. 1.   Micrographs of Drosophila polytene chromosomes
stained with racemic RuDIP by phase contrast (left) and
5   fluorescent (right) microscopy.   High concentrations of
RuDIP, bright regions in the fluorescent micrographs,
are found at the band regions (the dark regions by phase
contrast).

10   Fig. 2.   (a) Visible absorption spectra of racemic
$(phen)_3Ru^{2+}$ (50 μM) in the absence (—) and presence
(---) of DNA (1 mM).   (b) Luminescence spectra of free
(---) $(phen)_3Ru^{2+}$ and   of $\Lambda-(phen)_3Ru^{2+}$ (···)
and $\Delta-(phen)_3Ru^{2+}$ (—) in the presence of DNA (0.3 mM).
15   Ruthenium concentrations were 10 μM.   Sample excitation
was at 447 nm.

Fig. 3.   Relative mobilities of closed (●) and nicked
(○) circular pColEl DNA in the presence of increasing
20   concentrations   of   added $\Delta-$ (above   and $\Lambda-$ (below)
$(phen)_3Ru^{2+}$.   Bars indicate the width of the DNA bands
on the basis of the distribution of topoisomers.   Comi-
gration points of nicked and closed circular DNAs are
seen at 90 and 120 μM for the Δ and Λ isomers, respec-
25   tively.

Fig. 4.   Scatchard plot of $(phen)_3Ru^{2+}$ binding to calf
thymus DNA in buffer 1 at 22°C, where r is the ratio of
bound ruthenium to nucleotide concentrations and C is
30   the concentration of ruthenium free in solution.   The
solid curve represents the best fit to eq 1.

Fig. 5.   Circular dichroism of (□) $\Delta-(phen)_3Ru^{2+}$ and of
solutions after dialysis of $rac-(phen)_3Ru^{2+}$ against (+)
35   B-DNA and (■) Z-DNA.   Dialysis against B-DNA leads to
enrichment of the solution in the unbound Λ isomer.

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Fig. 6. Visible absorption spectra of racemic RuDIP
(5.3 μM) in the absence and presence of various concen-
trations of B-form calf thymus DNA (Left) and Z-form
poly[d(G-C)] (Right) in buffer 1.

5

Fig. 7. Luminescence spectra in buffer 2. ·····,
Racemic RuDIP (3 μM) free in solution; ---, racemic
RuDIP (3 μM) in the presence of Z-form poly[d(G-C)]; —,
B-form calf thymus DNA. Samples were excited at 482 nm.

10

Fig. 8. Relative change in absorption intensity at 460
nm of racemic RuDIP (x), Λ-RuDIP (O), and Δ-RuDIP (●)
at various DNA-phosphate/ruthenium ratios. Titrations
were conducted in buffer 1 using either B-form calf

15 thymus DNA (Upper) or Z-form poly[d(G-C)] (Lower).
Strong stereoselectivity with B-DNA is evident based on
the differential hypochromism seen between enantiomers,
whereas comparable titrations with Z-DNA show the same
hypochromic effects with each enantiomer. Because large

20 hypochromicity in the Λ isomer is seen only with Z-
DNA, Λ-RuDIP provides a probe for this conformation.

Fig. 9. (A) Cleavage of plasmid ColEl DNA in the pres-
ence of $(phen)_3Co^{3+}$ and light. The 1% agarose gel shows

25 the distribution of DNA forms (100 μM nucleotide) ini-
tially without cobalt (lane 1) and after irradiation at
254 nm (4-W mercury lamp) in the presence of 10 μM
$Co(phen)_3^{3+}$ for 0, 20, 40, and 60 min (lanes 2-5, left
to right). The samples were incubated in 50 mM Tris

30 acetate buffer, pH 7.0, and 18 mM NaCl and then electro-
phoresed for 1 h at 60 V and stained with ethidium. (B)
The cleavage is also stereoselective. Plasmid ColEl DNA
(100 μM) was incubated with Λ- (left) or $Δ-Co(DIP)_3^{3+}$

35 (right) (5 μM) and irradiated for 0, 0.5, 1.0, or 1.5 h
(lanes 1-4 and 5-8 for the Λ and Δ isomers, respective-

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ly). Incubation of this DNA with Λ-Co(DIP)$_3^{3+}$ in light has no effect, while incubation with Δ-Co(DIP)$_3^{3+}$ in light causes complete conversion of form I to form II.

5   Fig. 10. Plot showing the percent reduction in form I band intensity for pBR322 DNA (closed symbols) and pColEl DNA (open symbols) incubated with either Δ- (triangles) or Λ-Co(DIP)$_3^{3+}$ (circles) (5 μM) as a function of time irradiated. Extensive strand scission is
10   evident in pBR322 with both isomers.

Fig. 11. Experimental scheme used to map the single-stranded cleavage sites of Co(DIP)$_3^{3+}$.

15   Fig. 12. Densitometric scans of Λ-Co(DIP) fragmentation using the scheme depicted in Figure 1. The double stranded fragments produced in pBR322 using AvaI for linearization are the following sizes in kilobase pairs
20   (kb): 3.5 and 0.75, 2.9 and 1.5, 2.6 and 1.8. Note that these pairs of bands sum to 4.4 kb, the length of the plasmid. In pLP32, a pair of bands not present in pBR322 are seen at 3.3 and 1.1 kb. These bands indicate cleavage at the d(CG)$_{32}$ insert at position 375 (1.1 or
25   3.3 kb from the AvaI site at 1424). This plasmid was a gift of Dr. A. Nordheim.

Densitometric scans were performed on photographs using a Cary 219 spectrophotometer with gel scanning attach-
30   ment. Band sizes were quantitated using markers of known molecular weight. Note that since the bands were stained with ethidium bromide, the band intensities are weighted by their molecular weight.

35   Fig. 13. Coarse map of left-handed sites in pBR322. (a) Densitometric scans of Λ-Co(DIP)$_3^{3+}$ fragmentation

after linearization with EcoRI. Bands (in kilobase) are
produced at 3.3 and 1.1, 2.75 and 1.45, and 2.2. Since
EcoRI cleaves at position zero, these fragments corre-
spond to recognition sites at 3.3, 1.45, and 2.2.   (b)
5   Biological map of pBR322 showing positions of cleavage
by Λ-Co(DIP)$_3^{3+}$ (Λ).

Fig. 14.   Schematic view of the enantiomers of
(phen)$_3$Ru^{2+} bound to B-DNA, illustrating the basis for
10 stereo selectivity. Unfavorable steric interactions are
seen between the nonintercalated ligands of the Δ isomer
and the DNA phosphate backbone.   In contrast
the Δ isomer fits easily in a right-handed helical
groove.
15

Fig. 15.   Corey-Pauling-Kolton space-filling models
of Δ- and Λ-RuDIP with a right-handed B-DNA helix. The
orientation of the metal complex in the helix is shown
in the sketches. One ligand (not visible) is oriented
20 perpendicular to the helix axis for intercalation be-
tween the base pairs.   (A) Intercalation of
the Δ enantiomer.   The nonintercalated ligands fit
easily within the right-handed groove. (B) For
the Λ enantiomer, when one ligand is positioned for
25 intercalation, the remaining two ligands are blocked
completely above and below (arrows) by the right-handed
sugar-phosphate backbone, and this steric constraint
prevents ligand insertion between the base pairs. This
can also be seen by following the line of the DNA back-
30 bone, which, although completely visible in A, is inter-
rupted by the phenyl groups of Λ-RuDIP in B.

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Detailed Description of the Invention

As mentioned above, this invention concerns a coordina-
tion complex or salt thereof having the formula $(R)_3$---
5 Co(III), wherein R comprises 1,10-phenanthroline or a
substituted derivative thereof and R is bound to Co by
a coordination bond. A "substituted derivative" as the
phrase is used herein is a compound obtained by
replacing one or more hydrogen atoms present in 1,10-
10 phenanthroline with one or more moieties having the
characteristic that the complex containing the resulting
compound binds to DNA. Merely by way of example, the
substituted derivative of 1,10-phenanthroline may be
4,7-diamino-1,10-phenanthroline; 3,8-diamino-1,10-phe-
15 nanthroline; 4,7-diethylenediamine-1,10-phenanthroline;
3,8-diethylenediamine-1,10-phenanthroline;   4,7-dihy-
droxyl-1,10-phenanthroline; 3,8-dihydroxyl-1,10-phenan-
throline; 4,7-dinitro-1,10-phenanthroline; 3,8-dinitro-
1,10-phenanthroline; 4,7-diphenyl-1,10-phenanthroline;
20 3,8-diphenyl-1,10-phenanthroline;   4,7-dispermine-1,10-
phenanthroline, or 3,8-dispermine-1,10-phenanthroline.
Unless otherwise specified the complex of this invention
is a racemic mixture of enantiomers. The invention also
concerns the optically resolved delta and lamda isomers
25 of the complex.

One embodiment concerns a method for labeling DNA with a
complex of the formula $(R)_3$---M, wherein R comprises
1,10-phenanthroline or a substituted derivative thereof
30 as defined above, M comprises a suitable transition
metal, and R is bonded to M by a coordination bond. In
this and other embodiments a suitable transition metal
is one which is capable of forming an octahedral complex
with R, such as ruthenium (II) or cobalt (III). The
35 labeling method involves contacting the DNA with the

-15-

complex under suitable conditions such that the complex binds to the DNA, e.g. by intercalation.

5 The invention also concerns a DNA molecule labeled with a complex of the formula $(R)_3---M$, as defined above, and the complex is bound to the DNA, e.g. by intercalation. Preferably the labeled DNA molecule is produced by the above-described method.

10 A further embodiment is a method for selectively labeling Z-DNA with the lambda enantiomer of a complex of the formula $(R)_3---M$, as defined above. This method comprises contacting the DNA with the lambda enantiomer of the complex under suitable conditions such that the 15 complex binds to the Z-DNA. The invention further involves a labeled DNA molecule comprising Z-DNA and the lambda enantiomer of a complex of the formula $(R)_3---M$, as defined above, the complex being bound to the Z-DNA. Preferably the labeled DNA molecule is produced by the 20 above-described method.

Another embodiment of this invention concerns a method for detecting the presence of Z-DNA. This method involves selectively labeling Z-DNA according to the 25 above-mentioned method and detecting the presence of the complex bound to the Z-DNA, e.g. by spectroscopic methods (See Experiments hereinafter).

Still another embodiment of this invention is a method 30 for nicking double-stranded DNA by effecting single-stranded scission, i.e., breakage of at least one phosphodiester bond along the DNA. The method involves contacting the DNA with a cobalt (III)-containing complex of this invention under suitable conditions such 35 that the complex binds to the DNA, e.g. by intercala-

tion, to form an adduct and irradiating the adduct so
formed with a sufficient dose of ultraviolet radiation
of an appropriate wavelength so as to nick the DNA at
the site(s) of intercalation. An appropriate ultravio-
5 let wavelength in this an other embodiments is a wave-
length which is absorbed by the ligand bands of the
complex used. As described hereinafter, the ligand band
absorption of a complex of this invention may be deter-
mined spectroscopically by conventional methods.
10

This invention further involves a method for cleaving
double-stranded DNA which comprises nicking the DNA by
the above-mentioned method and treating the nicked DNA
so produced with a suitable enzyme capable of cleaving
15 single-stranded DNA under effective conditions to cleave
the nicked double-stranded DNA at the site of the
nick(s). By this method double-stranded scission of the
DNA is effected. Suitable enzymes for effecting double-
stranded cleavage of nicked DNA in this and other embod-
20 iments include those which are not deactivated in the
presence of the complex used for DNA nicking, e.g. Sl
nuclease.

An additional embodiment of this invention is a method
25 for selectively nicking Z-DNA by effecting breakage of
at least one phosphodiester bond along the Z-DNA. The
method involves contacting a DNA molecule containing a
Z-DNA sequence with the lambda enantiomer of a cobalt
(III)-containing complex of this invention under suita-
30 ble conditions such that the complex binds to the Z-DNA
to form an adduct and irradiating the adduct so formed
with a sufficient dose of ultraviolet radiation of an
appropriate wavelength, as previously defined, so as to
nick the DNA at the binding site(s). Double-stranded Z-
35 DNA may be selectively cleaved by selectively nicking

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the Z-DNA by the above-mentioned method and treating the
nicked DNA so produced with a suitable enzyme capable of
cleaving single-stranded DNA under effective conditions
to cleave the nicked DNA at the site of the nick(s).

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This invention further concerns a method for killing a
portion of a population of appropriate tumor cells. The
method involves contacting the tumor cells under suita-
ble conditions with an effective amount of the lambda

10 enantiomer of a coordination complex of the formula
$(R)^3$--M, as previously defined, to kill the tumor cells.
Alternatively, a racemic cobalt(III)-containing complex
of this invention may be similarly used. When a cobalt
(III)-containing complex is used, the method may further

15 comprise irradiating the tumor cells with a suitable
dose of ultraviolet radiation of an appropriate wave-
length at a suitable time after the tumor cells have
been contacted with the complex, permitting the complex
to nick DNA.

20

Still another embodiment of this invention is a pharma-
ceutical composition for the treatment of tumor cells in
a subject e.g. a human or animal. The pharmaceutical
composition comprises an effective anti-tumor amount of

25 the lambda enantiomer of a complex of the formula
$(R)_3$---M, as defined previously, and a pharmaceutically
acceptable carrier. Again, a racemic cobalt(III)-con-
taining complex may be used alternatively. Preferably
the complex is a cobalt(III)-containing complex. Suit-

30 able carriers include sterile saline or buffer-contain-
ing solutions or other carriers known in the art such as
those used with cisplatin.

Such a composition may be used in a method for treating
35 a subject, e.g. a human or animal, afflicted with tumor

cells so as to cause regression of the tumor cells. This method involves administering to the subject by a suitable route the pharmaceutical composition in an amount sufficient to cause regression of the tumor

5 cells. Suitable routes of administraiton include parenteral administration and topical administration, e.g. in cases such as skin cancers where the tumor cells are located on or near an exposed surface of the subject. Furthermore, if the complex used is a cobalt(III)-con-

10 taining complex, the method may additionally involve irradiating the tumor cells with a suitable dose of ultraviolet radiation of an appropriate wavelength permitting the complex to nick DNA. In this method hte irradiation should be conducted at a suitable time after

15 administration of the composition to the subject, i.e. to permit the complex to interact with the DNA. It should also be noted that optically resolved entantiomers of the complexes of this invention, specifically the lamboda isomer, may provide superior results both in

20 killing tumor cells and in treating subjects afflicted with tumor cells.

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Experimental Details

Octahedral complexes with three bidentate ligands like
phenanthroline do not contain an inversion center, and
therefore, as shown below, two enantiomeric forms are
present.   Note that the intercalating portion of the
molecule, the phenanthroline ligand, is coordinated
directly to the asymmetric center of the cation, the
metal.   Because this chiral metal center is really
proximal to the site of intercalation, the interaction
of these complexes with DNA provides a clear illus-
tration of stereospecific drug binding to a similarly
asymmetric DNA helix.   Furthermore this stereospecific
binding mode provides a means for designing probes for
DNA helicity.
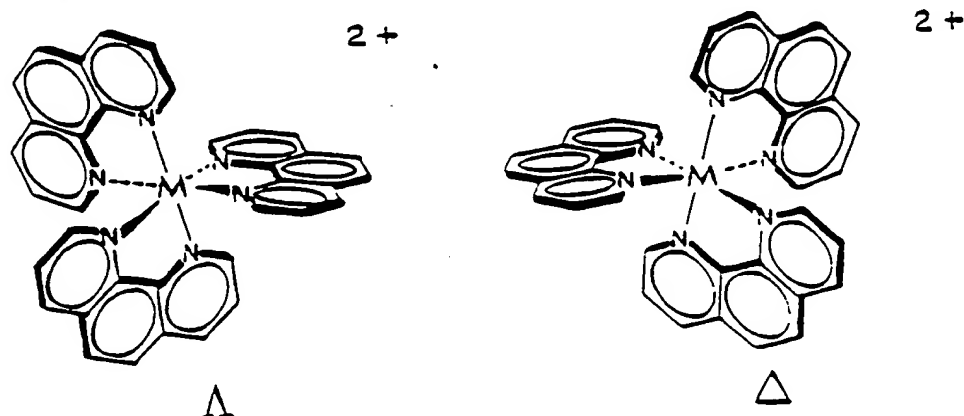
Ruthenium(II) complexes have been found useful because
of (i) the kinetically inert character of the low-spin
d^6species, (ii) their intense metal to ligand charge-
transfer (MLCT) band in the visible spectrum and since
(iii) many chemical and spectroscopic properties of the
poly(pyridine) complexes have been established.   The
electronic structure of the ground and excited states of
tris(bipyridine)ruthenium(II) has been examined in
detail.(45)   The strong visible absorption band, dis-
tinct from the absorption of DNA, in $(phen)_3Ru^{2+}$ as well

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as its high luminescence provide spectroscopic tools to
monitor the intercalative process.(38,46)   Most impor-
tantly, in contrast to (phen)$_3$Zn^{2+}, which is somewhat
labile,(47) the ruthenium(II) complexes are essentially
5   inert to racemization.(48)

Optical isomers of (phen)$_3$Ru^{2+} may be isolated in pure
form, (48,49) and the absolute configurations have been
assigned (50).   Electric dichroism measurements of
10   (phen)$_3$Ru^{2+} bound to DNA have been conducted (51) and
support the findings of enantiomeric selectivity.

Although a preference in binding is found between en-
antiomers in the phenanthroline series, both isomers do
15   in fact intercalate into the right-handed helix as
discussed above.   To amplify the chiral discrimination
and hence improve the sensitivity of the chiral probe,
suitable substituents, e.g. amino-, ethylenediamino-,
hydroxyl-, nitro-, phenyl- or spermine- substituents,
20   may be added to each phenanthroline ligand at appropri-
ate ring sites, e.g. 3,8- or 4,7. Bulky substituents at
the distal sites on the cation can block completely the
intercalation of the isomer into a right-handed he-
lix, and thus provides selective spectroscopic probes
25   for the handedness of the DNA duplex.   The structure of
the   left-handed   enantiomer   of   a   preferred   com-
plex,   Λ -tris(4,7-diphenyl-1,10-phenanthroline)rutheniu
m(II) (RuDIP), which binds to left-handed Z-DNA but not
to right-handed B-DNA, is shown below.
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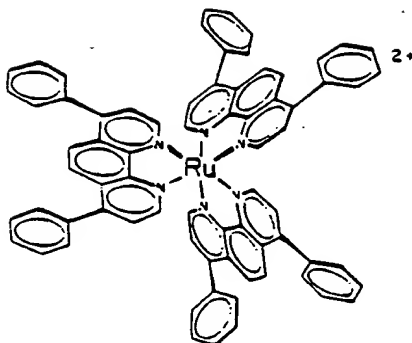
As for the smaller tris (phenanthroline) metal complex-
es, DNA binding is evident in the presence of racemic
RuDIP.(52)　The similarity to $(phen)_3Ru^{2+}$ in spectral
characteristics on binding to DNA, i.e. hypochromicty
and luminescence enhancements, suggested that RuDIP
also binds to the duplex by intercalation.　RuDIP is
however substantially more bulky and hydrophobic than
the parent $(phen)_3Ru^{2+}$ cation.　Substitution of phenyl
groups at the distal 4- and 7- positions leads to sever-
al significant perturbations.　First, the solubility of
the ruthenium complex in aqueous solution is diminished
appreciably, which is a practical consideration.　Sec-
ond, stacking with the base pairs in the helix now
requires that the phenyl groups rotate into the plane of
the phenanthroline.　The rotation to planarity can be
accomplished with minimal steric interactions of the
neighboring hydrogen atoms by lengthening the carbon-
carbon bond between the phenyl and phenanthroline moie-
ties; precedence for this type of structural distortion
is found in the case of biphenyl which is planar when
stacked in a solid state lattice. (53)　The phenyl
groups once rotated into the plane of the phenanthroline
increase the surface area for base pair overlap substan-
tially as compared with $(phen)_3Ru^{2+}$, stabilizing the
intercalatively bound metal-DNA complex.　An increased

affinity for the helix is indeed apparent in gel ele-
ctrophoresis experiments where changes in supercoiled
DNA mobility are first evident at an order of magnitude
lower total ruthenium concentration than for
5    $(phen)_3Ru^{2+}$. Hydrophobic interactions of the non-inter-
calated ligands abutting the helical groove may also
account for some increase in affinity for the duplex.
Third, and perhaps most importantly, the added phenyl
substituents increase the chiral discrimination marked-
10   ly. While the delta isomer can still bind closely into
the right-handed helix, intercalation of the lambda
isomer is now completely blocked.

Because the steric constraints are governed by the
15   helicity of the duplex, RuDIP enantiomers can offer a
specific chemical probe to distinguish right-handed and
left-handed DNAs. While for the lambda isomer steric
interactions between the non-intercalated phenyl groups
and the DNA-phosphate backbone prevent close association
20   to B-DNA, no similar repulsive interactions limit bind-
ing to the left-handed Z-DNA.(16) Hence an assay of
duplex binding by the ruthenium enantiomers equivalently
assays the DNA conformation. It is necessary at this
point to note that RuDIP is indeed a probe for helical
25   structure and does not itself promote a conformatioion
transition between B- and Z-DNA. The circular dichroic
spectra of poly(dGC) in the B-form or Z-form without
ruthenium present are identical to those of DNA solu-
tions containing ruthenium at an added ratio of 0.05 per
30   nucleotide. Unlike other smaller intercalators which in
binding at high drug/DNA ratios cause Z--B conforma-
tional transitions (54,55,56), racemic RuDIP does not
interconvert the helical forms. The enantiomers of
RuDIP provide, therefore, a chemical means to examine
35   DNA conformation and specifically, the handedness of the

helix. DNAs of particular repetitive sequence or those
covalently bound by particular drugs will be intriguing
to study. Because of the high luminescence of the
ruthenium complexes, some interesting applications
5　become feasible. Figure 1 shows, for example, fluores-
cent micrographs(57) of Drosophila polytene chromosomes
stained with racemic RuDIP. These samples were prepared
in collaboration with Dr. O.J. Miller and Dr. D. Mill-
er. Centers of high RuDIP concentration correlate
10　closely with the band regions seen by phase contrast and
no staining is evident in either interband regions or
puffs. Experiments using the individual enantiomers can
be conducted to determine whether a high local concen-
tration of a particular conformation is present. These
15　micrographs should provide a useful addition to those
obtained through staining with fluorescent antibodies,
(31) with the particular advantage of pointing out
specific structural features depending upon the stereo-
selectivity observed.
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Metallointercalation reagents also offer flexibility in
the design of a stereospecific DNA nicking agent. Such
agents have been prepared by using tris-phenanthroline
complexes with a suitable choice of redox-active metal,
25　e.g. cobalt. Tris(phenanthroline)cobalt(III) (58), for
example, at low concentrations cleaves DNA when irradi-
ated at 254 nm. Furthermore the high stereospecificity
of the tris(diphenylphenanthroline) (DIP) metal isomers
(59) with DNA helices is preserved in these cleavage
30　reactions.

Numerous spectroscopic and x-ray crystallographic stud-
ies have shown that DNA may adopt a range of conforma-
tions, from the right-handed A- and B-forms to the
35　striking left-handed Z-DNA helix (60,61). Regions of

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conformational heterogeneity along the strand, such as cruciform structures, single-stranded loops, and left-handed segments, have been detected using DNA enzymes (62), and it has been suggested that local DNA conformation might play some role in regulating gene expression. Chiral metal complexes of this invention can intercalate into the helix and are therefore particularly advantageous in probing local DNA conformation (15,34,52,64). Tris(diphenylphenanthroline) (DIP) ruthenium(II) complexes provide a spectroscopic probe for helix handedness; the lambda isomer, which does not bind B-DNA owing to steric constraints, binds avidly to Z-DNA (63). Upon photoactivation, the analogous cobalt isomers, $Co(DIP)_3^{3+}$, furthermore cleave DNA stereospecifically, providing a sensitive assay for local regions in the Z-form (64). The specific left-handed sites have now been mapped where Λ-$Co(DIP)_3^{3+}$ cleaves in the plasmids pLP32 (29), containing a $d(CG)_{32}$ insert, and pBR322 (65). In pLP32 a primary cleavage occurs at the insert, and in native pBR322 cleavage occurs at four discrete sites: 1.45 kb, 2.3 kb, 3.3 kb, and 4.2 kb. These sites correspond to segments of alternating purine-pyrimidines. Moreover, these positions map to the ends of the three distinct coding regions in pBR322: the tetracycline resistance gene, the origin of replication, and either end of the ampicillin resistance (β-lactamase) gene. The locations of these left-handed segments suggest that Z-DNA might serve as a conformational punctuation mark to demarcate the ends of genes.

Λ-Co(DIP)$_3{}^{3+}$, the photoactivated DNA cleaving agent used here to detect Z-DNA segments is shown below.

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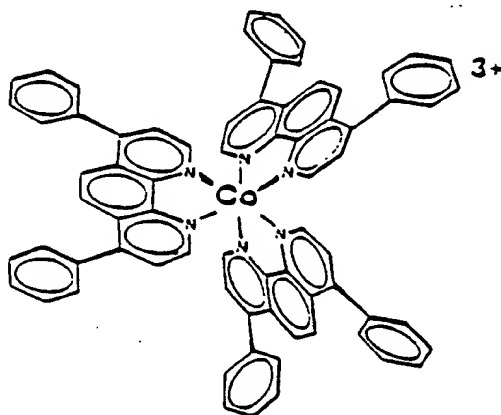
The possible application of the complexes of this invention in chemotherapy has also been investigated. Beyond the antimicrobial and antitumor activities common to intercalating agents, if Z-DNA is important in gene regulation, the stereospecific intercalators of this invention could display a unique potency _in vivo_. Preliminary experiments in tissue cultures have shown high toxicity without irradiation, and phototherapy may provide greater potency and tissue specificity.

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Experiments

Materials and Methods

5   I.   Tris(phenanthroline)ruthenium II

Ruthenium Complexes.   [(phen)$_3$Ru]Cl$_2$:2H$_2$O was prepared as follows: to a solution of 0.5 g K$_2$RuCl$_5$ in 50 ml hot water containing 1 drop 6N HCl was added 0.81 g phenan-
10   throline monohydrate (Aldrich). The resulting mixture was boiled for 15 minutes to fully dissolve the ligand and thereafter 0.75 ml of 50% hypophosphorous acid neutralized with 2N NaOH was added. The solution was refluxed for 30 minutes and filtered hot to remove solid
15   material. To the filtrate was added 10 ml 6N HCl, the volume reduced to about 20 ml and cooled at 0°C over-
night.   Single orange crystals of [(phen)$_3$Ru]Cl$_2$ were obtained. See also (38). Enantiomers were obtained by successive diastereomeric recrystallizations with anti-
20   mony D-tartrate anion.(49) At most, two recrystalliza-
tions were required to achieve $[\alpha]_D$ 1317, after which point additional purification did not yield increased optical activity. Several samples of the Δand Λ isomers were used in the course of the various binding studies,
25   and, on the basis of comparison to literature values for the specific rotation, all showed a level of optical purity given by [(C(Δ-Ru)-C(Λ-Ru)/(C(Δ-Ru) + C(Λ-Ru))]≥.92. The steroisomers were isolated as perchlor-
ate salts; and elemental analyses (performed by
30   Galbraith Lab., TN) were as follows: %C, 49.34; %H, 3.29; N, 9.52; calculated for [(phen)$_3$Ru] (ClO$_4$)$_2$.2H$_2$O, %C, 49.32; %H, 3.22; %N, 9.59. Spectrophotometric and luminescence titrations of racemic Ru(phen)$_3$Cl$_2$ and
35   equimolar mixtures of Δ - and Λ -Ru(phen)$_3$(ClO$_4$) with DNA agreed closely, indicating that the presence of

perchlorate ($\leq$50 M) was without effect. Stock ruthenium solutions were either freshly prepared or kept in the dark. Concentrations of $(phen)_3Ru^{2+}$ were determined spectrophotometrically by using $\varepsilon_{447}1900$ M^{-1} cm^{1}.(38)

BUFFERS AND CHEMICALS. Experiments were carried out at pH 7.1 in buffer 1 (5 mM Tris, 50 mM NaCl), buffer 2(5mM Tris, 4.0 M NaCl), or buffer 3 (50 mM Tris acetate, 20 mM sodium acetate, 18 mM NaCl pH 7.0). Solutions were prepared with distilled deionized water. Plasticware was used throughout and was cleaned by soaking overnight in 10% HNO_3 followed by exhaustive rinsing. Dialysis membranes were prepared by the following protocol: After they were boiled successively in sodium carbonate, 1% EDTA, and 1% SDS and exhaustively rinsed in deionized water, the membranes were heated to 80°C in 0.3% sodium sulfite, acidified at 60°C with 2% sulfuric acid, and thereafter rinsed again with deionized water and 1% EDTA. This procedure serves to minimize metal binding to the membranes.

Nucleic Acid. Calf thymus DNA, obtained from Sigma Chemical Co., was purified by phenol extraction as described previously.(9c) Poly(dGC).poly(dGC) from P.L. Biochemicals Inc. and plasmid ColEl from Sigma Chemical Co. were extensively dialyzed in buffer before use. DNA concentrations per nucleotide were determined spectrophotometrically by assuming ε_{260} 6000 M^{-1} cm^{-1} for calf thymus DNA and ε_{260} 8400 M^{-1} cm^{-1} for poly(dGC).

Spectroscopic Measurements. Absorption spectra were recorded on a Cary 219 spectrophotometer. Absorbance titrations of racemic, Δ-and Λ-$(phen)_3Ru^{2+}$ in buffer 1 were performed by using a fixed ruthenium concentration to which increments of the DNA stock solution were

added. Ruthenium was also added to the DNA stock to keep the total dye concentration constant. Luminesence measurements were conducted on a Perkin-Elmer LS-5 flourescence spectrophotometer at ambient temperature.

5   Samples were excited at 447 nm, and emission was observed between 500 and 700 nm. All experiments were carried out in buffer 1 with $(phen)_3Ru^{2+}$ concentrations typically of 10 μM and DNA phosphate/ruthenium ratios varying from 1 to 50. Lifetime measurements were per-

10   formed on an Ortec 776 single-photon counter and timer in line with an Apple Computer. The samples were excited with a PRA 510A nanosecond lamp, and emission was observed at 593 nm. Reproducible lifetimes for the bound species in the presence of free ruthenium were

15   obtained   by   neglecting   the   first $1.2 \, \mu s \, (2\tau [Ru(phen)_3^{2+}{}_{free}}$, of the decay curve.

Electrophoresis. Dye gel electrophoresis of supercoiled DNA in 1% agarose was performed in buffer 3 by using the

20   method of Espejo and Lebowitz (6) modified as described previously.(15) Ruthenium concentrations in the gels were carefully determined on the basis of several absorbance readings of the stock concentrations for the enantiomers. Because of the high background lumines-

25   cence of $(phen)_3Ru^{2+}$, gels were destained for 24 hours in buffer prior to staining with ethidium.

Equilibrium Dialysis. Binding isotherms were obtained on the basis of dialysis of calf thymus DNA in buffer 1

30   against $(phen)_3Ru^{2+}$ at 22°C. The DNA was dialyzed first exhaustively in buffer to remove small fragments. Thereafter, dialysis against ruthenium was allowed to continue for at least 24 hours after which time equilibration was achieved. Each sample consisted of 2mL of

35   dialysate containing Δ-,Λ-, or rac-$(phen)_3Ru^{2+}$, varying

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in concentration between 50 and 1,000 μM, and, within
the dialysis bag, 1 mL of 1 mM DNA phosphate. To de-
termine bound and free concentrations, absorbance spec-
tra were taken of dilutions (3-50 μM). Free ruthenium
concentrations outside the bag were determined on the
basis of absorbance readings at 447 nm. For concentra-
tions of ruthenium inside the bag, in the presence of
DNA, readings were obtained at the isosbestic point,
where ε_{464} 13630 M^{-1} cm^{-1} (vide infra). Equilibrium
dialysis of poly (dGC) was conducted similarly in buffer
2. Measurements of circular dichroism were obtained on
a Jasco J-40 automatic recording spectropolarimeter.
Because of the irregular baseline of the instrument, all
spectra were digitized and replotted after base-line
subtraction. Data analyses were performed on a IBM PC
and a Digital VAX 11/780.

II.   Tris(4,7-diphenyl-1,10-phenanthroline)rutheniumII

Nucleic Acids. Calf thymus DNA (Sigma) was purified by
phenol extraction (66). Poly[d(G-C)] (P-L Biochemi-
cals) was dialyzed at least three times before use.
Experiments were conducted at pH 7.2 in buffer 1 [4.5 mM
Tris·HCl/45 mM NaCl/150 μM Co$(NH_3)_6$Cl$_3$/10% dimethyl
sulfoxide], buffer 2 [5 mM Tris·HCl/50 mM NaCl/150 μ M
Co$(NH_3)_6$Cl$_3$], or buffer 3 [5 mM Tris·HCl/4.0 M NaCl].
DNA concentrations per nucleotide were determined spec-
trophotometrically assuming ε_{260} = 6600 $M^{-1}·cm^{-1}$ for
calf thymus DNA (67) and ε_{260} = 8400 $M^{-1}·cm^{-1}$ for
poly[d(G-C)] (68). In preparing Z-DNA, poly[d(G-C)]
stock solutions weree incubated in the cobalt hexammine
buffer for 2-18 hr to ensure both a complete transition
to the Z conformation and minimal aggregation. Stock
solutions were examined spectrophotometrically and by CD
before use.

Ruthenium Complexes. The synthesis of RuDIP trihydrate
was carried out as described above, substituting 4,7-
diphenyl-1,10-phenanthroline for the unsubstituted 1,10-
phenanthroline. See also (38). Concentrations were
5   determined spectrophotometrically using ϵ_{460} = 2.95 x
10^4 $M^{-1} \cdot cm^{-1}$. Elemental analyses were consistent with
literature values. The Δ and Λ isomers were either
separated by successive recrystallizations with the
antimony tartrate anion in 50% ethanol or prepared by
10   asymmetric synthesis in the presence of antimony tar-
trate and then recrystallized. Λ -RuDIP forms the less
soluble diastereomeric salt with antimonyl D-tartrate.
The separated isomers were isolated finally as perchlo-
rate salts. The assignments of absolute configuration
15   have been made on the basis of the relative binding
affinities of these enantiomers for B-DNA (see below).
Many rounds of recrystallization yielded a small quanti-
ty of Λ-RuDIP having $[\theta]_{283}$ = -4.0 x 10^3 deg· $M^{-1} \cdot cm$.
This assignment is consistent with both the UV CD for
20   tris(1,10-phenanthroline)ruthenium(II)   [(phen)$_3$Ru^{2+}],
assigned previously (31), and studies of the enantiomer-
ic preference of (phen)$_3$Ru^{2+} for B-DNA (34, 35, 51).
The optical purities of the Δ - and Λ-RuDIP samples used
below were 41% and 70%, respectively. Therefore the
25   sample designated Δ-RuDIP contains 70.5% Δ isomer and
29.5% Λ isomer, and that designated Λ-RuDIP is composed
of 14% Δ- and 86% Λ-RuDIP.

Spectroscopic Measurements. Absorbance spectra were
30   recorded using a Varian Cary 219 UV/visible spectropho-
tometer and luminescence spectra, with a Perkin-Elmer
LS-5 fluorescence spectrophotometer. Titrations were
carried out using a constant ruthenium concentration (4-
6 μM) to which increments of either calf thymus DNA or
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solubility in aqueous solution (≤ 10 μM), dimethyl sulf-
oxide was included in buffer 1. CD spectra of B-DNA or
Z-poly[d(G-C)] with 150 μ M $Co(NH_3)_6^{3+}$ were unaffected by
the presence of the dimethyl sulfoxide. Although more
difficult, titrations in buffer 2 and buffer 3 were also
conducted.

III.   CobaltIII Complexes

Tris(4,7-diphenyl-1,10-phenanthroline)cobalt(III)
$(Co(DIP)_3^{3+})$ tri-tartrate was prepared as follows: 4,7-
diphenyl-1,10-phenanthroline (Aldrich) was dissolved in
a minimum volume of ethanol to which one third stoichio-
metric $CoCl_2 \cdot 6H_2O$ was added. The green brown solution
was oxidized by using Br_2/H_2O, and a heavy orange pre-
cipitate formed immediately. The solution was refluxed
for 1 h, and concentrated hydrochloride was added. The
bromine oxidation was then repeated. The crude chloride
salt was used directly for enantiomeric separations.
With either l- or d-tartaric acid (Aldrich), the deep
red tartrate (Tar) diastereomeric salts $[\Lambda-Co(DIP)_3] \cdot (L-$
$Tar)_3$ and $[\Delta-Co(DIP)_3 \cdot (d-Tar)_3$, were prepared by succes-
sive recrystallizations in 50% ethanol, pH 7.0.

Chemical and spectroscopic data for these complexes are
as follows: Anal. Calcd for $[Co(DIP)_3](Tar)_3 \cdot H_2O$
$(CoC_{84}N_6O_{19}H_{65})$ C, 66.32; H, 4.32; N, 5.52; Found: C,
65.87; H, 4.46; N, 5.78.   Absorption spectra
showed λ_{max} at 278 and 312 nm (shoulder). The circular
dichroic spectra resemble those of enantiomers of
$Ru(DIP)_3^{2+}$, and absolute configurations have been as-
signed on that basis.

IV. Cleavage Methods

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[Co(DIP)$_3$](tartrate)$_3$ (10 µM) was added to pBR322 DNA
(100 µM nucleotides) in 50 mM tris-acetate buffer con-
taining 18 mM NaCl, ph 7.0. The 20 µl sample was then
irradiated at 315 nm (with a 1000 W Hg/xenon lamp nar-
5   rowed to 315 ± 5 nm with a monochrometer) for 90 seconds
and ethanol precipitated. The ethanol wash removes
unreacted Co(DIP)$_3^{3+}$ as well as the metal and ligand
products of the reaction. After resuspension in tris-
acetate buffer containing 50 mM NaCl and 10 mM MgCl$_2$, pH
10 7.0, restriction enzyme was added (either EcoRI, BamHI,
AvaI or NdeI) using at least a threefold excess to
insure complete linearization. This was incubated at
37°C for 45 min. The pH of the reaction mixture was
then lowered to 5.0 and 10 mM Zn(NO$_3$)$_2$ added along with
15 4 units of S1 nuclease, and the samples were incubated
for 5 min at 37°C. This step causes cleavage of the DNA
by S1 opposite the site nicked by Co(DIP)$_3^{3+}$. Electro-
phoresis on 1% agarose gels followed (50 mM tris-ace-
tate, 18 mM NaCl, pH 7.0) to resolve the double stranded
20 fragments produced. In these experiments pBR322 se-
quences are numbered beginning at the EcoRI site accord-
ing to Sutcliffe (65). Gels were stained with 5 µg/ml
ethidium bromide for 0.5 hr then destained in buffer for
2 hr. Gels were photographed using a Polaroid 600
25 camera with a red filter and 615 positive/negative film
and irradiated from below.

V.   In vitro Screening

30 For cell culture studies, a modification of the tech-
niques of Fischer (69) was used. The cells were incu-
bated in McCoy's Medium 5A with 15% fetal calf serum.
The initial inoculum was 40,000 to 60,000 leukemic
cells/ml. For studies of the inhibition of cell growth,
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0.1 ml of a 20-fold concentration of the drug in ques-
tion was added to 2 ml of media containing 4×10^4
cells/ml in Linbro tissue culture multiwell plates and
allowed to incubate at 37° in 5% CO_2 for 96 hr. By
5   these times, growth to approximately 10^6 cells/ml oc-
curred in the control wells. The contents of each well
were agitated to resuspend the cells and counted on a
Coulter Counter.   The percentage of inhibition of
growth and the concentrations inhibiting cell growth by
10   50% were calculated.   Cell culture experiments were
conducted with mouse leukemia cell lines L1210 and P815.
The cell lines and growth medium may be obtained from
the American Type Culture Collection (ATCC), Rockville,
Maryland.

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Results

I.   Tris(1,10-phenanthroline)ruthenium (II)

5   Spectroscopic Studies

The binding of Λ- and Δ-(phen)$_3$Ru^{2+} to duplex DNa leads
to a decrease and small shift in the visible absorption
of the ruthenium species and a corresponding increase
10   and shift in luminescence. Figure 2 shows both the
absorption   spectra   and   luminescence   spectra   of
(phen)$_3$Ru^{2+} in the presence and absence of calf thymus
DNA.   The spectral changes observed here are often
characteristic of intercalation.

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The hypochromic shift in the broad charge-transfer band
of (phen)$_3$Ru^{2+} as a result of binding to the polynucleo-
tide can be seen in Figure 2A.   A decrease of 12% in
absorbance at 447 nm is found for the saturating DNA
20   levels employed.   Since at these concentrations 70%
(phen)$_3$Ru^{2+} is in the bound form, ϵ(bound)/ϵ(free) =
0.83 at 447 nm.   The hypochromic effect is small com-
pared with that found for other intercalators, which may
indicate that the charge is not being preferentially
25   localized onto the intercalated ligand.   Also for the
free ruthenium complex the predominant polarization of
the charge-transfer band is perpendicular to the molecu-
lar C3 axis (46) rather than parallel to the intercala-
tive plane. Shown in the figure is the change in absor-
30   bance for the racemic mixture; also because the observ-
able hypochromic effect is small, significant differ-
ences between enantiomers were not obtained.   For both
isomers a spectral shift of 2 nm to lower energy is
found, which supports an electronic stacking interaction
35   of the phenanthroline ligand with the base pairs of the

helix. Isosbestic points at 355 and 464 nm are also
apparent.

An enhancement in the luminescence of $(phen)_3Ru^{2+}$ on
binding to duplex DNA parallels the observed hypochromi-
city. Figure 2B shows the emission spectra of free
$(phen)_3Ru^{2+}$ and of both lambda and delta isomers bound
to DNA. These spectra also reveal a shift of 2 nm to
longer wavelength with DNA binding. Moreover, in the
presence of 0.25 mM DNA phosphate, emission increases of
48% and 87% are observed respectively for Λ -
and $\Delta-(phen)_3Ru^{2+}$ (10 μM). Note that a significant
fraction of the ruthenium is free in the presence of
the DNA at these concentrations, but the associated
increase in solution viscosity for higher DNA concen-
trations precluded studies at saturating binding levels.
The greater increase in luminescence seen for
the Δ isomer in the presence of DNA over that for
the Λ isomer indicates simply that a higher proportion
of the Δ isomer is bound, rather than that their modes
of association with the helix differ. Measurements of
the excited-state lifetimes of enantiomers in the ab-
sence and presence of the DNA yielded results consistent
with this interpretation. For $\Delta-$ and $\Lambda-(phen)_3Ru^{2+}$,
determined separately and as a racemic mixture, identi-
cal experimental lifetimes of 2.0 and 0.6 μs were found
respectively in the presence and absence of DNA. Both
isomers therefore bind to the helix in a similar fash-
ion, and indeed, if fully bound, would display similar
enhancements in luminescence. Substantial increases in
fluorescent lifetimes with intercalation have been
observed in several instances (2-4,70), notably for
ethidium, and may be explained by the greater rigidity
and lower collisional frequency of the molecule when
stacked within the helix.

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Measurements of Helical Unwinding

Both Λ - and Δ-(phen)$_3$Ru^{2+} reversibly unwind and rewind
supercoiled DNA as a function of increasing concentra-
5   tion of ruthenium(II), and for a given total concentra-
tion, a greater unwinding effect is evident for
the Δ isomer.   Figure 3 illustrates the migration of
pColEl DNA through 1% agarose gels containing increasing
levels of (phen)$_3$Ru^{2+}.   Mobilities are plotted relative
10 to the supercoiled DNA control to permit the inclusion
of data from several gel electrophoresis trials.   As can
be seen in the figure, both isomers unwind the helix.
With increasing levels of ruthenium bound, the duplex
unwinds, and for a closed circle this unwinding results
15 in first a release of negative supercoils at low levels
bound and then the introduction of positive supercoils;
the nicked DNA, without similar topological constraints,
displays no variation in mobility.   The bars in the
figure indicate the width of the DNA bands, which vary
20 because of the distribution of topoisomers in the sam-
ple.   The observed duplex unwinding provides a strong
indication of intercalative binding.   Control experi-
ments also show the unwinding to be reversible; prein-
cubation of the DNA with ruthenium complex has no effect
25 on gel mobility.   It is interesting to note that no DNA
cleavage is observed as a result of binding (phen)$_3$Ru^{2+},
even after irradiation with ultraviolet light (short
wavelength) for 1h.

30
For a given level of total ruthenium, a higher amount of
duplex   unwinding   is   found   in   the   presence
of Δ-(phen)$_3$Ru^{2+}, respectively.   The comigration   of
nicked and closed circular DNAs occurs in the presence
of 90 and 120 μM Δ-(phen)$_3$Ru^{2+}.   This comigration point
35

represents a fixed amount of helical unwinding. A lower
added concentration of $\Delta-(phen)_3Ru^{2+}$ is needed to unwind
all the negative supercoils in the pColEl DNA. These
results therefore also reflect the higher affinity of
the Δ isomer over the Λ isomer for the right-handed
helix. At a given total concentration of ruthenium,
more of the Δ isomer is bound and greater helical un-
winding is evident. The alternative explanation for the
lower concentration of the Δ isomer at the comigration
point would be that the Δ isomer has a larger unwinding
angle than the Λ isomer, so that the Δ isomer unwinds
the duplex more per drug bound. A particularly large
difference between unwinding angles (about 30%) would be
needed to account for the electrophoresis results,
however, and only small variations in unwinding angles
are generally observed (4). Moreover larger, if any,
structural perturbations should accompany binding of
the Λ isomer to the right-handed helix rather than
the Δ isomer. Here then, as well, the results show that
the Δ isomer possesses a greater affinity for the DNA
duplex.

These data may be used to estimate the intercalative
unwinding angle. If we assume for the racemic mixture
that the average comigration point of nicked and closed
forms occurs with 100 μM ruthenium, then, on the basis
of our determination of the binding constant (vide
infra), a binding ratio of 0.089 per nucleotide is
required to unwind fully the supercoils in the plasmid.
Interestingly this value is identical with that calcu-
lated for ethidium, since in buffer 3 the comigration of
nicked and closed pColEl species in the presence of 5 x
10^{-7} M dye was observed. Therefore the unwinding angle
for $(phen)_3Ru^{2+}$ is estimated to be the same as that for
ethidium (6).

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Equilibrium Dialysis Experiments

The results of dialysis of calf thymus DNA with racemic $(phen)_3Ru^{2+}$ at 22°C in buffer 1 are shown in Figure 4 in the form of Scatchard plot (71). The data have been fit
5 by nonlinear least-squares analysis to the following equation governing noncooperative binding to the helix, as derived by McGhee and von Hippel (72):

$$\frac{r}{C_F} = \frac{K(O)}{2}(1 - 2Lr)\left(\frac{1 - 2Lr}{1 - 2(L-1)r}\right)^{L - 1} \tag{1}$$

10

where r is the ratio of the bound concentration of ruthenium to the concentration of DNA phospate, C_F is the concentration of ruthenium free in solution, K(O) is the intrinsic binding constant, and the integer I, which
15 measures the degree of anticooperativity, is the size of a binding site in base pairs. In fitting the data, the binding parameter K(O) was varied for several integer values of L. The best fit, shown as the solid curve in Figure 4, yielded a binding constant K(O) = 6.2×10^3
20 M^{-1} (±2%) and an exclusion site size (L) of four base pairs. Data from luminescence titrations were consistent with this curve. The binding constant is quite low in comparison to values of 3×10^5 and 5×10^4 M^{-1} (extrapolated to the ionic strength of our buffer) for
25 ethidium and $[(phen)Pt-(en)]^{2+}$, respectively (73). The lower affinity of $(phen)_3Ru^{2+}$ is not surprising since only partial stacking of the phenanthroline ligand is feasible in this octahedral complex; greater overlap of the phenanthroline with the base pairs may be achieved
30 in the square-planar platinum(II) species. The steric bulk of the nonintercalated ligands determines also the large four base-pair site size compared to a two base-pair (neighbor excluded) site for basically planar reagents (9,74). Inspection of space-filling models
35

show that the perpendicular phenanthroline ligands each
span two base pairs either above or below the intercala-
tively bound phenanthroline, which is consistent with
the binding isotherm.

5   In these equilibrium dialysis experiments of the racemic
mixture, the relative binding of the two enantiomers to
the polynucleotide may be determined sensitively on the
basis of the degree of optical enrichment of the unbound
enantiomer in the dialysate. Figure 5 shows the circu-
10   lar dichroism (1.5×10^{-5} M) of the dialysate after
equilibration of calf thymus DNA (1 mM) with racemic
(phen)$_3$Ru^{2+} (2×10^{-4} M). Also shown for comparison is
the circular dichroism of Δ-(phen)$_3$Ru^{2+} (0.2 µM). The
spectra show clearly that the dialysate has been opti-
15   cally enriched in the less favored isomer.
The Δ enantiomer binds preferentially to the right-
handed helix. The degree of chiral discrimination may
be made more quantitative by comparing the level of
optical enrichment (2% for the sample shown) with the
20   overall amount of ruthenium bound. On the basis of a
simple competition between the enantiomers for sites
along the helix with no cooperativity and, for simplifi-
cation, describing the binding by each enantiomer in
terms of the familiar Scatchard equation, X, the ratio
25   of binding constants K(Δ)/K(Λ) may be calculated as
follows:

$$X = (PK(\Delta)(n - r) + 1)\frac{(C_B + \Delta C)}{(C_B - \Delta C)} - PK(\Delta)(n - r) \qquad (2)$$

30   where P is the concentration of DNA phosphate, n is the
ratio of drug to DNA phosphate bound at saturation,
taken as 0.125, C_B is the total concentration of ruthe-
nium bound, and ΔC is the difference in free concentra-
tions between Δ and Λ isomers as measured by the inten-
35

-40-

sity in the circular dichroism. Measurements of several samples yielded values of 1.1-1.3 for X. Thus the binding affinity of Δ-(phen)$_3$Ru^{2+} is found to be 10-30% greater than Λ-(phen)$_3$Ru^{2+} for calf thymus DNA by this

5   method. This value is comparable to the differences seen in luminescence and unwinding experiments. Since the overall binding of (phen)$_3$Ru^{2+} is small, binding isotherms obtained through equilibrium dialysis tended to show some scatter. A direct comparison of the bind-

10   ing isotherms for the enantiomers in equilibrium dialy- sis experiments using the pure isomers therefore could not be achieved; significant differences were not evi- dent. Interestingly it appears that the method of optical enrichment yields the most sensitive assay for

15   the differential binding.

Since the enrichment experiment provides the most sensi- tive method to examine enantiometric discrimination, poly(dGC) in 4M NaCl was also dialyzed against rac-

20   (phen)$_3$Ru^{2+} to test for any enantiomeric preferences in binding to a left-handed DNA helix (16,17). At the low binding levels examined, the circular dichroism of the polymer remains inverted, indicating that racemic (phen)$_3$Ru^{2+} did not induce a Z-- B transition. After

25   equilibrium dialysis with bound concentrations compara- ble to those in earlier experiments using calf thymus DNA, e.g., under conditions where low levels of enrich- ment could be detected, no optical activity was observed in the dialysate. Therefore, although intercalative

30   binding had occurred, given similar spectral character- istics as in binding to the right-handed helix, no preference in binding was evident. In Figure 5 the essentially base line spectrum of a solution after dialysis against Z-form poly(dGC) has also been includ-

35   ed. This lack of discrimination for (phen)$_3$Ru^{2+} is

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understandable in view of the shallow, almost grooveless
character of the left-handed Z-DNA helix.

II.   Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II)

5

In spectroscopic studies of racemic RuDIP with B- and
Z-DNA, changes are seen in both the visible absorption
and luminescence spectra of RuDIP on addition of either
B- or Z-form DNA.   Hence, binding may be monitored
10   sensitively   using   either   spectroscopic   technique.
Visible absorption titrations of racemic RuDIP in buffer
1 (described below) with calf thymus DNA and Z-form
poly[d(G-C)] are shown in Fig. 6. The overall similari-
ty of these titrations is apparent.   Binding of either
15   duplex DNA leads to hypochromicity in the intense metal-
to-ligand charge-transfer band of the ruthenium complex.
A small red shift (about 2 nm) in the spectrum of the
bound complex and an isosbestic point at 485 nm can be
seen.   That spectral changes occur as a function of
20   addition of either DNA form suggests that racemic RuDIP
binds to both B- and Z-DNA.   The similarity in spectral
changes most likely reflects a similar mode of associa-
tion of the ruthenium complex with either the right-
handed B-DNA helix or the left-handed Z-DNA helix.

25

Differences in binding to the two forms are evident,
however. A greater reduction in the absorption intensi-
ty of Ru-DIP accompanies binding to Z-form poly[d(G-C)]
than to the B-DNA helix.   In Fig. 6, for example, the
30   apparent reduction in intensity with the addition of a
13:1 ration of calf thymus DNA-phosphate/ruthenium is
only 9% whereas, for the left-handed helix, the reduc-
tion occurring at a nucleotide/ruthenium ratio of 5:1 is
17%.   The greater hypochromicity in binding to Z-DNA is
35   explained in part by the different stereoselectivities

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governing binding to each helix. Although both enantio-
mers bind to Z-DNA, only the Δ enantiomer may bind
easily to right-handed B-DNA. The differences in ste-
reoselectivity cannot fully account for the difference
in hypochromicity, however, because the hypochromicity
in spectra of racemic RuDIP with Z-DNA is more than
twice that observed with calf thymus DNA. If one as-
sumes that the extinction coefficients for RuDIP when
bound to each helix are the same, which seems reasonable
based on the equal isosbestic points observed, then the
larger hypochromic effect with Z-DNA suggests that both
RuDIP enantiomers possess a greater affinity for Z-form
poly[d(G-C)] than for calf thymus DNA. Equilibrium
dialysis experiments support this conclusion.

The luminescence of RuDIP is also enhanced on binding to
the DNA duplex. Fig. 7 shows the emission spectrum of
racemic RuDIP (3 μM) in the absence and presence of calf
thymus DNA and Z-form poly[d(G-C)] (15 μM nucleotide).
The shift in the spectrum to lower energy is particular-
ly pronounced despite the broad nature of the transi-
tion; the maximum shifts 10 nm to longer wavelength in
the presence of DNA. Greater luminescence is seen here
on binding to B-DNA, despite the lower apparent affinity
for this helix. In buffer 2, at a
DNA-phosphate/ruthenium ratio of 5:1, the emission
intensity of racemic RuDIP increases by 30% and 47% in
the presence of Z-form poly[d(G-C)] and calf thymus DNA
respectively. The different enhancements may depend in
part on nucleic acid composition as well as duplex
conformation, RuDIP bound to B-form poly[d(G-C) yields
less luminescence than when bound to calf thymus DNA,
despite having an equal affinity for these polynucleo-
tides.

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Racemic RuDIP appears to bind to both B- and Z-DNA rather than promoting a transition from one conformation to the other. CD spectra of Z-form poly[d(G-C)] in $Co(NH_3)^{3+}$ (buffer 1) or in 4 M NaCl (buffer 3) are
5   unaltered by the addition of racemic RuDIP at a nucleotide/ruthenium ratio of 10. Conversion of the Z-form to B-form with RuDIP is inconsistent also with the differential hypochromism and luminescence observed. If RuDIP promoted a Z to B transition, albeit inefficiently,
10 rather than binding to both B- and Z-helices, then both the reduction in absorbance and the enhancement in luminescence observed on addition of Z-DNA would be less than or equal to that found with B-DNA, i.e., in proportion to the fraction of DNA converted. Instead signifi-
15 cantly greater hypochromism is found when Z-DNA rather than B-DNA is added to the racemic mixture or indeed to each enantiomer individually. Therefore racemic RuDIP must bind to both DNA comformations. Consistent with these results, a conformational transition from Z-DNA to
20 B-DNA would not be expected if the affinity of the metal cation for the Z-form were greater than that for the B-form. The ethidium cation, which binds to B-DNA by intercalation, is known to promote a Z to B transition at high binding ratios (75,76) and presumably possesses
25 a greater affinity for the B-form helix. The substantially larger RuDIP cation cannot saturate the DNA to comparable levels, which may be an important distinction. Moreover, although the ethidium ion can fully intercalate into B-DNA, RuDIP cannot and the noninter-
30 calating ligands of RuDIP may dominate its interactions with the duplex.

The utility of the RuDIP enantiomers as a probe for helical conformation becomes apparent when the binding
35 characteristics of each enantiomer to B- and Z-DNAs are

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compared. Plots of the relative absorbance at 460 nm of the individual enantiomers as a function of the addition of either B-form calf thymus DNA or Z-form poly[d(G-C)] in buffer 1 are shown in Fig. 8. Based on the presence

5   or absence of hypochromicity, it is clear that although RuDIP binds to B-DNA, the Λ isomer does not.   Δ RuDIP does however bind to Z-DNA. Indeed, with Z-DNA no stereospecificity is observed. Hence the assay of duplex binding by the Λ isomer yields a sensitive

10   assay for the Z-DNA conformation.

Strong enantiomeric selectivity governs the interaction of RuDIP with the right-handed B-DNA helix. The decrease in absorbance with increasing DNA concentration

15   observed for the Λ, racemic mixture, and Δ samples can be fully accounted for based on the percentage of the Δ enantiomer present in the particular preparation (see Experimental). The pure Λ enantiomer does not bind to B-DNA. The presence of the phenyl groups at the

20   4 and 7 positions of the nonintercalated phenanthroline ligand has served to amplify the chiral discrimination. In comparison, differences in binding of $(phen)_3Ru^{2+}$ enantiomers had been seen only in spectrophotometric titrations at high DNA/ruthenium levels; the ratio of

25   the affinities for B-DNA of Δ to Λ isomers is about 1.3(34,35). For RuDIP, with hydrogen atoms now replaced by phenyl groups, instead of simple interference with the DNA phosphate oxygen atoms, one finds that the steric bulk of the phenyl groups completely blocks

30   interactions of the isomer in the right-handed groove. Δ-RuDIP, however, binds with facility to a right-handed helix, indeed more avidly than Δ - $(phen)_3Ru^{2+}$. This striking amplification in enantiomeric selectivity for RuDIP compared with $(phen)_3Ru^{2+}$

35   strongly supports our model for stereospecific intercalation.

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Z-DNA serves as a poor template to discriminate between the enantiomers; identical reductions in absorbance intensity are found for the Δ and Λ isomers (Fig.8). Because of the shallow and very wide character of the
5　major groove in Z-DNA, there are no steric constraints comparable with that found with B-DNA. Hence, if the binding modes are equivalent, no chiral specificity would be expected. The similarity in spectral characteristics of RuDIP in binding to the different DNA
10　duplexes points to this similarity in binding modes. However, the lack of chiral specificity in binding to Z-DNA does limit what can be said at present about the interaction of RuDIP enantiomers with a Z-form helix. Based on relative hypochromicities, it appears that
15　both Λ and Δ-RuDIP possess greater affinities for Z-form poly[d(G-C)] than for B-DNA. Hydrophobic interactions with the helical surface may lend some stability to the bound complex(77). The difference in affinity furthermore does not reflect a preference for base composition.
20　Titrations of racemic RuDIP with B-form poly[d(G-C)] in buffer 1 lacking cobalt hexammine showed hypochromicity equal to that seen with calf thymus DNA. Also, cobalt hexammine itself does not appear to alter binding to the helix. RuDIP titrations using calf thymus DNA with and
25　without $Co(NH_3)_6^{3+}$ were identical. In addition, the interaction cannot be explained purely be electrostatic interactions. Although smaller, hypochromic effects, approximately one-third of that shown here, are found in titrations in 4 M NaCl (buffer 3) with either poly[d(G-
30　C)] or calf thymus DNA. Partial intercalation into the DNA by both RuDIP enantiomers would be consistent with these results. It is finally important to note that the similar titrations of both enantiomers that are seen with Z-DNA but not with B-DNA suggest that neither
35

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enantiomer converts the Z-form helix to the B-DNA con-
formation. If that were the case, selectivity between
the enantiomers would become apparent.

5   III.   <u>Cobalt(III) Complexes</u>

Figure 9 shows gel electrophoretic separations of
plasmid ColEl DNA after incubation with cobalt complexes
and irradiation for variable times. DNA cleavage is
10   followed by monitoring the conversion of supercoiled
(form I) closed circular plasmid DNA to the nicked
circular form (form II) and linear (form III) species .
(The original ColEl preparation contained 60% form I and
40% form II molecules.) Figure 9A reveals the complete
15   conversion of form I to II after a 1-h irradiation in
the presence of 10   M (phen)$_3$Co^{3+}. Neither irradiation
of the DNA at these low intensities without cobalt nor
incubation with cobalt without light yielded significant
strand scission. (Irradiation at 310nm where there are
20   strong ligand transitions also leads to cleavage). It
is likely that the reduction of Co(III) is the impor-
tant step leading to DNA cleavage and not that irradia-
tion provides a means to generate cobalt(II) in situ.
DNA incubation with the tris(phenanthroline) complex
25   initially in the cobaltous form yielded no reaction
unless irradiated. Presumably the cobaltous complex is
oxidized in solution to the cobaltic species, since it
is the +3 oxidation state in cobalt polyamine complexes
that is photochemically active. Also dithiothreitol
30   inhibits activity of Co(phen)$_3$$^{3+}$, perhaps by precluding
regeneration of an active cobalt(III) species. This
finding is in contrast to the iron and copper systems
where thiols are thought to stimulate activity by gener-
ating the metal species in the reduced form (39-43).
35   Interestingly, electrophoresis also reveals with in-

creasing irradiation a small reproducible increase in the mobility of form II; this may reflect some short-range radical-induced DNA cross-linking (78).

5 The cleavage reaction is furthermore strongly stereo-specific. Figure 1B shows pColEl DNA of low superhe-lical density after incubation with either Λ-Co(DIP)$_3^{3+}$ or Δ-Co(DIP)$_3^{3+}$ (17,18) and irradiation with ultravio-let light. Incubation of pColEl DNA of low superhelical
10 density with the Λ isomer, which cannot bind to a right-handed duplex owing to steric constraints, yields no appreciable reaction (19), whereas incubation with Δ-Co(DIP)$_3^{3+}$, which is able to associate closely with right-handed B-DNA, shows efficient nicking activi-
15 ty comparable to that seen with Co(phen)$_3^{3+}$. Nicking was observed, however, upon titration of pColEl of increasing superhelical density with ΛCo(DIP)$_3^{2+}$. This different cleavage efficiency by each enantiomer is consistent with the earlier finding (7) of conforma-
20 tional discrimination by the ruthenium(II) isomers; one enantiomer of Ru(DIP)$_3^{2+}$ binds to B-DNA, but both Δ- and Λ-Ru(DIP)$_3^{2+}$ bind to the left-handed Z-DNA helix. These results underscore the importance of an intimate association of the metal with the duplex.
25

In Figure 9B the overall concentrations of the cobalt isomers are equal, yet the Λ trication, if it cannot intercalate, does not yield DNA strand scission.

30 The Λ-tris(phenanthroline) metal complexes do, however, bind to left-handed Z-DNA (21,22). We examined the plasmid pBR322 containing a 42-base pair alternating guanine-cytosine insert (pLP42) (29,79) and which was shown (28,17) to adopt the Z-conformation in 4 M NaCl.
35 Under these conditions, cleavage by both Co(DIP)$_3^{3+}$

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enantiomers is obtained. Hence the isomer may recog-
nize and cleave left-handed helices. More interesting,
however, is the finding, plotted in Figure 10 as percent
loss of supercoiled form, that the plasmid pBR322 at
5   physiological salt concentrations and without extreme
superhelix underwinding also is significantly cleaved by
the isomer. Given our other results of differential
binding based on DNA helicity (35,63) and the differen-
tial cleavage of ColEl of low superhelical density
10 described above, it appears that Λ-Co(DIP)$_3$$^{3+}$ might
bind to and cleave a natural left-handed segment in
pBR322 DNA of low superhelical density in normal salt
concentrations. These observations support the find-
ings by Rich and co-workers (80) of anti-Z antibody
15 binding to the 14-base pair alternating purine-
pyrimidine segment in pBR322). The statistically sig-
nificant 14 base pair sequence (CACGGGTGCGCATG) in
pBR322 shows alternation of purine and pyrimidine with
one base out of register. Alternating purine-pyrimidine
20 sequences tend to adopt the Z conformation. The plasmid
pColEl sequence contains no comparable stretch of alter-
nation.

IV. Cleavage Site Mapping
25

Irradiation at 315 nm of Λ-Co(DIP)$_3$$^{3+}$ (10 μM) solutions
containing supercoiled pLP32 or pBR322 yields nicked
circular form II DNAs. Photoreduction of Co(DIP)$_3$$^{3+}$
enantiomers bound to DNA leads to oxidative single-
30 strand scission at the DNA binding site (64). In order
to establish that cleavage and therefore binding occurs
at discrete sites, the scheme outlined in Figure 11 was
employed. Following irradiation of Co(DIP)$_3$$^{3+}$-DNA
samples and the production of nicked circles, the DNAs
35 were linearized using a restriction enzyme which is

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known to cleave the plasmid at only one site along the
strand. Subsequent treatment with S1 nuclease, which is
specific for single-stranded regions, cleaves the DNA
only opposite to the cobalt-induced nick producing a
5 pair of linear fragments. From the sizes of these
fragments, determined based upon their gel electro-
phoretic mobilities relative to markers, the distance of
the cleavage site from the restriction site origin may
be obtained. In order to distinguish whether the site
10 is clockwise or counterclockwise to the origin, at least
two restriction enzymes which cut at sufficiently dis-
tinct locations were examined. It is important to
notice that this procedure yields distinct fragments
only if binding and subsequent cleavage occurs at dis-
15 crete sites. Non-specific cleavage produces fragments
of all sizes and hence a smear on the gel; thus the
presence of some contaminating form II DNA just alters
the background intensity. Control experiments of sample
irradiated without cobalt or cobalt binding but without
20 irradiation yielded no distinct bands. Non-specific DNA
damage as a result of irradiation was negligible.
Controls showed that full linearization of the plasmid
was essential, however, to avoid mapping S1 hypersensi-
tive sites (62). Some restriction enzymes did not yield
25 complete linear digests, either because of thymine dimer
formation at the restriction site or inhibition due to
$Co(DIP)_3^{3+}$ reaction, and these were not used. Finally,
samples were irradiated only for short times so that no
more than one nick per plasmid would occur. The fact
30 that the sizes of pairs of fragments must sum to 4363
base pairs provided a useful experimental redundancy.
By this general procedure the coarse map of Λ-$Co(DIP)_3^{3+}$
cleavage sites in any plasmid may be obtained.

35

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The plasmid pLP32 which contains a Z-DNA segment at a well-defined location (29,81) was examined first. The plasmid had been constructed by inserting a $d(CG)_{32}$ fragment into the filled-in BamHI site (position 375) of pBR322 (29). The densitometric scan of the AvaI digest after reaction with Λ-Co$(DIP)_3^{3+}$ is shown in Figure 12. In addition to the linear form several bands and shoulders are evident; their sizes in kilobase pairs (kb) are indicated in the figure. The appearance of the pair of fragments at 3.3 kb and 1.1 kb from the AvaI site (position 1424) shows that a major cleavage point is indeed at the Z site. Parallel digestion with NdeI established this position uniquely. More interesting perhaps is the comparison to the AvaI digest of pBR322, the same plasmid but lacking the insert. The pattern here is identical except that it lacks the 3.3 kb and 1.1 kb fragments. In pLP32, then, the cobalt complex must recognize and cleave a site not present in pBR322, the Z-form $d(CG)_{32}$ insert. The result demonstrates that the complex can cleave specifically at a left-handed site.

Other conformations are not similarly accessible to the chiral cobalt complex. Λ-tris(diphenylphenanthroline) complexes of ruthenium(II) and cobalt(III) do not react as assayed spectrophotometrically (34,63) (for ruthenium) and by cleavage assays (64) (for cobalt) with B-form helices. The delta isomer in contrast can bind both B- and Z- forms and photolysis experiments using Δ-Co$(DIP)_3^{3+}$ show non-specific cleavage of the linear DNA but with some specific band formation. It has also been found that $(phen)_3Ru^{2+}$ complexes do not bind significantly to double-stranded RNA and hence it is unlikely that Λ-Co$(DIP)_3^{3+}$ would recognize an A-form helical conformation. Additionally, racemic Co$(DIP)_3^{3+}$ cleavage of single-stranded phage DNA X174 was exam-

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ined. Here after photolysis about 12% cleavage was observed, less than the 15% double-stranded content in the X174 sample, calculated based upon hypochromicity. It is unlikely then that $Co(DIP)_3^{3+}$ enantiomers could

5   recognize open looped regions of a plasmid. Instead the only DNA conformation for which appreciable binding and cleavage by $\Lambda-Co(DIP)_3^{3+}$ has been found is Z-DNA.

Photolysis and digestion of both pLP32 and pBR322 actu-
10   ally yields several distinct fragments, seen in Figure 12, and therefore additional cleavage sites for $\Lambda-Co(DIP)_3^{3+}$, similar structurally to the left-handed $d(CG)_{32}$ insert must be present in these plasmids. Based upon numerous trials using either EcoRI, BamHI,
15   AvaI, or NdeI for linearization, there appears to be four discrete cleavage sites in pBR322, given in order of intensity $1.45 \pm .05$ kb, 3.3 ± 0.1 kb $> 4.24 \pm 0.02$ kb $> 2.25 \pm .07$ kb. The standard deviations are based upon averaging at least seven experiments. The plasmid
20   pLP32 shows cleavage at these same positions in addition to cleavage at the insert. Figure 13A shows a typical EcoRI digest. Fragment pairs are evident and, since EcoRI linearizes at the origin, the lengths of one fragment of the pair shows the position in kilobases of
25   the site. The relative intensities, weighted by the fragment molecular weight, reflects either the relative site affinity for $\Lambda-Co(DIP)_3^{3+}$ or relative cleavage efficiency at a site. Variations in relative site intensities as a function of irradiation time and also
30   as a function of salt concentration in the incubation mixture were observed. The influence of salt and super-helical density on the relative expression of these sites is currently being examined. The weakest site recognized is consistently at 2.3 kb. Table 1 summa-
35   rizes the specific sites in pBR322 found with cleavage by $\Lambda-Co(DIP)_3^{3+}$.

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Table 1
Λ-Co(DIP)$_3^{3+}$ Cleavage Sites

	Alternating Purine-Pyrimidine Sequences
1.45 ± 0.05 kb	1447-1460 CACGGGTGCGCATG
2.25 ± 0.07 kb	2315-2328 CGCACAGATGCGTA
3.32 ± 0.11 kb	3265-3277 GTATATATGAGTA
4.24 ± 0.02 kb	4254-64 TCCGCGCACAT

V. In vitro Screening

Diphenyl tris complexes of this inventions were screened for cytotoxic activity against mouse leukemia cells as previously described. The results for two of the complexes are set forth in Table 2.

Table 3. Cytotoxicity of Cobalt and Ruthenium Complexes

Compound	Cell Line[1]	ID$_{50}$ (µg)/ml)
Ru(DIP)$_3$Cl$_2$	L 1210	4.49
	P 815	5.42
Co(DIP)$_3$· (d-tartrate)$_3$	L 1210	0.51
	P 815	0.52

[1] L 1210 and P 815 are mouse leukemia cell lines, see Burchenal, J.H. et al., CANCER RESEARCH, 42:2598-6000 (July 1982)

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Discussion

I. Tris(phenanthroline)ruthenium(II)

5   Results of experiments described above indicate that
tris(phenanthroline)ruthenium(II) binds to DNA by inter-
calation.   The optical changes on binding to DNA agree
with those seen for other intercalators.   Hypochromi-
city in the metal to ligand charge-transfer (MLCT) band
10   is observed and represents an overall 17% decrease in
intensity.   Stacking interactions with the base pairs
lead to hypochromic shifts in the $II \rightarrow II^*$ transitions
of organic intercalating dyes, and it is interesting
that the II symmetry of the MLCT preserves the hypo-
15   chromic effect.   Substantial increases in the lumines-
cence of $(phen)_3Ru^{2+}$ also accompany binding to the
duplex.   The enhancement in emmission and corresponding
increased luminescent lifetimes may simply reflect the
decreased mobility of the complex when sandwiched into
20   the helix.   Emission lifetimes are comparable to those
found for $(phen)_3Ru^{2+}$ in sodium lauryl sulfate micel-
les.(82)   In addition to perturbations in the elec-
tronic structure of the bound reagent, intercalation
leads to hydrodynamic changes in the DNA duplex.   With
25   increasing concentrations, $(phen)_3Ru^{2+}$ reversibly un-
winds and rewinds superhelical DNA.   Although not abso-
lutely definitive,(4) this result provides a very
strong indication of intercalation.   Surely helical
unwinding and lengthening accompany the binding of
30     $(phen)_3Ru^{2+}$.   Finally the binding isotherms obtained by
equlibrium dialysis yield parameters that are reasonable
for the intercalative mode of association.   The complex
binds to duplex DNA with relatively low affinity and,
when bound, encompasses a four base-pair site.   The
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octahedral coordination around the metal precludes effective stacking of the complex between base pairs. If the complex is viewed with one of the three phenanthroline ligands inserted into the helix, then the other two ligands actually protrude above and below the face of this phenanthroline and decrease the effective area of overlap. Hence only partial insertion is possible, which accounts for the low binding constant. The fact that so small a region of overlap with only partial insertion is necessary for a stabilizing interaction with the duplex is interesting to consider with respect to the binding of aromatic amino acid residues to DNA. The four base-pair site size is similarly consistent with the structural model for the bound complex, presented herein, where one ligand intercalates and the remaining two ligands span the groove of the helix. A site size of four base pairs is understandable since the internuclear distance of 10.4 $\overset{*}{}$A between distal hydrogen atoms on the ligands not only exceeds the 10.2 A of a single interbase pair site but must result in partial blockage of the next neighboring base pair both above and below the plane of insertion.

Furthermore the direct comparison between enantiomers of spectroscopic features, binding properties, and structural parameters establishes that the Δ enantiomer possesses the greater affinity for a right-handed helix. Intercalation of tris(phenanthroline)ruthenium(II) into the duplex imposes different steric constraints on Δ and Λ isomers, and it is this difference that determines the enantiomeric selectivity. Perhaps the strongest evidence in support of intercalation is the observed chiral discrimination. The Δ enantiomer, a right-handed propeller-like structure, displays a greater affinity than Λ-(phen)$_3$Ru^{2+} for the right-

handed DNA helix. Figure 14 illustrates the basis for
the enantiomeric selectivity. With one phenanthroline
ligand intercalated, the two nonintercalated ligands of
the Δ isomer fit closely along the right-hand helical
groove. The nonintercalated ligands of the Λ enantio-
mer, in contrast, are repelled sterically by the phos-
phate backbone of the duplex. The disposition of the
left-handed enantiomer is opposed to the right-handed
helical groove. The stereoselectivity seen here is in
the direction proposed originally for $(phen)_3Zn^{2+}$ and
supports the assignment of the absolute configurations
for the zinc enantiomers. No stereoselectivity is
apparent in the association of $(phen)_3Ru^{2+}$ with Z-DNA.
This left-handed helix does not contain a groove of
size and depth comparable to that in B-DNA, and there-
fore comparable or actually mirror image steric con-
straints are not expected. Instead the base pairs in
the Z-DNA helix are pushed outward toward the solvent,
resulting in at most a very wide and shallow major
"groove". Hence Z-DNA provides a poor template for this
discrimination.

Although it is $\Delta-(phen)_3Ru^{2+}$ that binds preferentially
to B-DNA, the Λ enantiomer does intercalate into the
right-handed helix. The ratio of affinities
of Δ and Λ isomers for B-DNA is 1.1-1.5, depending upon
the method of analysis. Luminescence enhancements and
unwinding experiments with supercoiled DNA suggest
the Δ isomer to bind 30-50% more strongly. It is inter-
esting that supercoiling does not alter the selectivi-
ty. Optical enrichment assays, which can be extremely
sensitive and reflect a direct competition between
enantiomers for the helix, yield values of 10-30%
greater affinity of $\Delta-(phen)_3Ru^{2+}$ for calf thymus DNA.
A more precise determination of relative affinities is

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difficult because the binding constant of either enan-
tiomer for the helix is low. In fact, then, the enan-
tiomer can bind to the right-handed helix, although the
phosphate backbone limits access. The addition of
5   bulky sustituents onto the phenanthroline rings, in
severly blocking interactions of the left-handed enan-
tiomer with the duplex, is necessary to prevent com-
pletely intercalation of the Λ isomer.

10 These results provide an example of stereospecific
interactions with DNA. The stereoselectivity observed
is governed by the handedness of the DNA helix. The
asymmetric duplex structure serves as a template which
discriminates in binding the small molecules on the
15 basis of their chirality. It is interesting that the
change in symmetry of the metal complex alone yields a
significant difference in its recognition by the helix.
The comparison of spectroscopic and binding character-
istics of isomers of $(phen)_3Ru^{2+}$ has afforded a detailed
20 description of the structural basis for the enantiomeric
selectivity observed first for $(phen)_3Zn^{2+}$ (15). The
difference   in   biological   activities   of
tris-(phenanthroline)metal enantiomers is, perhaps, also
a function of this stereoselectivity.(83) Indeed the
25 interaction of $(phen)_3Ru^{2+}$ with DNA illustrates how
stereospecificity may be incorporated into the design of
drugs that bind to the duplex and provides a means to
design reagents that can distinguish the handedness of
the DNA helix.(35,63,64) Certainly these stereospe-
30 cific interactions underscore the ability of small
intercalating drugs to recognize differences in nucleic
acid structure.

II.   <u>Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II)</u>
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The chiral DIP complexes, as shown by the experiments described above serve as specific chemical probes for the handedness of the DNA helix in solution. Spectrophotometrics titrations have shown that, although one RuDIP enantiomer, assigned as Λ-RuDIP, does not bind at all to the B-DNA helix, the bulky asymmetric cation can bind to Z-DNA. Monitoring the binding of this isomer to DNA by any means therefore equivalently assays the helical conformation. The intense metal-to-ligand charge-transfer band in the ruthenium complexes provides a particularly sensitive handle with which to examine the binding, either spectrophotometrically or through its accompanying luminescence.

Striking enantiometic selectivity is found in the interactions of the RuDIP cations with right-handed B-DNA, and this chiral discrimination is consistent with an intercalative model. The changes in the visible spectrum of RuDIP, i.e., the hypochromic shift and luminescence enhancement observed in the presence of duplex DNA parallel in detail those seen in spectra of $(phen)_3Ru^{2+}$ as a function of DNA addition. It has been shown that $(phen)_3Ru^{2+}$ binds to B-DNA by partial intercalation of the phenanthroline ligand between the base pairs (34,35). Given that generally the ruthenium-metal-to-ligand charge-transfer transition shows little sensitivity to solvent or environment (84,85), the close resemblance of properties of RuDIP to $(phen)_3Ru^{2+}$ suggests that the cations bind the DNA in a similar fashion. Intercalation of the diphenylphenanthroline ligand between the helix base pairs requires that the phenyl groups rotate into the plane of the phenanthroline ligand. This rotation to a planar structure with minimal steric interactions between nearby hydrogen atoms can be accomplished by legthening the carbon-carbon bond

between the phenyl and phenanthroline moieties. Equiva-
lent structural distortions are seen in biphenyl, which
is planar in the stacked solid lattice (86). Also, the
extremely bulky tetrapyridyl-porphyrin cations, which
5 require extensive distortation, are thought to bind to
the DNA duplex by intercalation (87, 88). Importantly,
once rotated into the plane of the phenanthroline, the
phenyl groups in RuDIP add substantially to the surface
area available for overlap with the base pairs as com-
10 pared with $(phen)_3Ru^{2+}$, and therefore greater stability
of the bound ruthenium-DNA complex is expected. In
fact, binding of RuDIP to DNA assayed by any method
becomes evident at 10% of the concentration of
$(phen)_3Ru^{2+}$, which reflects the increased affinity of
15 RuDIP over $(phen)_3Ru^{2+}$ for B-DNA. Perhaps the clearest
support for the intercalation model rests in the dramat-
ic enhancement in stereoselectivity observed for RuDIP
in comparison with $(phen)_3Ru^{2+}$. The phenyl groups,
while facilitating intercalation of Δ-RuDIP into the
20 right-handed helix, completely preclude binding by
the Λ enantiomer.

Corey-Pauling-Kolton space-filling models of the RuDIP
complexes with a B-DNA helix are shown in Fig. 15. The
25 orientations with respect to the helix are indicated in
the accompanying sketches. The Λ enantiomer, with one
diphenylphenanthroline ligand intercalated, can fit very
closely along the helical groove. The two noninterca-
lating ligands, with a disposition in line with the
30 right-handed helix, abut the helical groove. These
close hydrophobic interactions of the nonintercalated
ligands are not possible with the mirror-im-
age enantiomer. In contrast, as presented in Fig.14,
if one ligand (not visible) is oriented perpendicular to
35 the helix axis, then the two remaining ligands of

the Λ enantiomer are disposed contrary to the right-
handed groove. The ruthenium model must therefore be
shown in front of the DNA helix in the figure, rather
than intercalated, because the interaction of the phenyl
5   groups with the DNA-phosphate backbone at the positions
indicated by the arrows completely blocks access. Thus,
the stereoselectivity that we see is determined by the
steric   constraints   imposed   by   the   asymmetry   in   the
helix, its handedness.

10

Just as the helix asymmetry can serve as a template to
discriminate   between   RuDIP   enantiomers,   differential
binding by the enantiomers may be used advantageously in
determining the chirality of the helix. Table 3 indi-
15   cates a general scheme to probe helical conformations
using RuDIP cations. Although Λ-RuDIP does not bind to
the right-handed B-DNA duplex, spectrophotometric titra-
tions have shown significant binding to Z-DNA and there-
fore hypochromism of Λ-RuDIP on addition of a test DNA
20   sample may be used as an indication of the Z-conforma-
tion.   It was particularly interesting to us to find
that no stereoselectivity governs binding to the Z-form
helix.   The bulky cation likely avoids the very narrow
helical crevice in the Z-DNA structure, and intercala-
25   tive binding to the more shallow hydrophobic surface in
Z-DNA, the equivalent of the major groove in the B-form,
would not be expected to yield any chiral discrimina-
tion.   Z-DNA does not mirror B-DNA in solution.   In-
stead we predict that a left-handed but more. B-like
30   conformation (18, 89, 90) would yield a mirror-image
selectivity.

35

Table 3. Scheme for probing DNA conformation with RuDIP enantiomers

Reactivity		
With the Δ isomer	With the Λ isomer	DNA duplex conformation
+	-	Right-handed B-like
+	+	Left-handed Z-like or lacking a groove
-	-	Unstacked or with base pairs inaccesible
-	+	Left-handed B-like

The chiral tris(diphenylphenanthroline) metal complexes may therefore be used in solution to examine DNA helical conformations: those of naturally occurring sequences, in the presence of drugs, and in protein-bound complexes. Furthermore, the reagents represent a new route for conformation-specific drug design.

III. Cobalt(III) Complexes

The DNA cleavage experiments described above are important in several respects. The photoactivated DNA cleavage reaction with $Co(phen)_3^{3+}$ illustrates with a simple inorganic complex the notion of DNA strand scission mediated by a locally generated redox reaction. Reduction of Co(III) with perhaps concomitant hydroxide oxidation may be responsible for cleavage. With regard to applications, this photoactivated reaction should make possible "footprinting" as a function of time. Most importantly, the differential cleavage of ColEl DNA by enantiomers of $Co(DIP)_3^{3+}$ represents a clear example of a conformation-specific DNA cleaving molecule. This molecule will be useful in determining regions of Z-DNA conformation within long segments of native DNA. More-

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over the high level of recognition of DNA conformation
by these chiral inorganic complexes suggests the power-
ful application of stereospecificity in DNA drug design.

5   IV. Cleavage Site Mapping

Features unique to the plasmid recognition sites deter-
mined above with Λ-Co(DIP)$_3{}^{3+}$ were exam-
ined. Λ-Co(DIP)$_3{}^{3+}$ is not a sequence-specific reagent
10   and there is no sequence homology evident at these
positions in pBR322. Instead Λ-Co(DIP)$_3{}^{3+}$ is a confor-
mation-specific cleavage agent and it is the common
left-handed conformation at these locations that is
likely to be recognized by the cobalt complex. Alter-
15   nating purine-pyrimidine sequences have been shown to
adopt the Z-DNA conformation most readily, because
alternative residues in Z-DNA have bases in the syn
conformation. Inspection of the pBR322 sequence re-
vealed that the Λ-Co(DIP)$_3{}^{3+}$ recognition sites included
20   the longest runs of alternating purines and pyrimidines
allowing for one base out of alternation. Table 1 shows
also the alternating sequences that appear within one
standard deviation of each measured recognition site.
At positions 1447, 2315, 3265, and 4254 begin respec-
25   tively 14, 14, 13, and 11 base pair regions with alter-
nating purine and pyrimidine residues having one mis-
take. These regions correspond essentially to one heli-
cal turn in a Z-DNA conformation and are the longest of
such conformation homology within the plasmid. Sequenc-
30   es not recognized by Λ-Co(DIP)$_3{}^{3+}$ were then considered.
There are several other sequences, beginning at 1171,
1533, and 1709, that also constitute 11 base pair seg-
ments with one mistake that are not significantly
cleaved by Λ-Co(DIP)$_3{}^{3+}$, and the longest sequence of
35   alternation in the plasmid with no mistakes, 10 bp

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beginning at position 2785, is also not cleaved. At
this stage it is not known whether the flanking sequenc-
es at these sites are affecting Z-DNA formation
or Λ-Co(DIP)$_3^{3+}$ recognition. The sequences within the
recognition sites detected do have a range of GC con-
tents. It is interesting that the Z-DNA conformation
in pBR322 has been detected at the 1447 site in equally
low salt buffers by a completely different route, cross-
linking studies (80) with anti-Z-DNA antibodies, which
lends confirmation to the Z-form assignment. Thus it
is proposed that these four sites of alternating purine-
pyrimidine residues adopt the Z-conformation under
physiological conditions in native supercoiled pBR322
and are specifically recognized and cleaved
by Λ-Co(DIP)$_3^{3+}$.

It is interesting, finally, to ask whether these Z-DNA
segments share some common biological function in this
plasmid. pBR322, assembled from three naturally occur-
ring plasmids, contains three genetically distinct
coding regions, the tetracycline resistance genes,
the β-lactamase gene conferring ampicillin resistance,
and the origin of replication. Figure 13B shows the map
of these genes in pBR322. It is curious to notice the
correspondence in position between the ends of these
discrete coding elements and the Z-DNA recognition
sites. A single polypeptide in pBR322 appears to be
necessary for tetracycline resistance (91). The 3'-end
of the region encoding this peptide is thought to be
near the AvaI site at 1425 bp; sequences upstream from
the tetracycline resistance promotor (which begin at 45
bp) were lost in construction from pSC101.
The β-lactamase gene is defined upstream by the start
site at 4201 with the -35 consensus region for the
promoter ending at 4236, 18 bp away from the Z-form

cleavage site (92). The 3'-end of the region encoding β-lactamase is found at position 3295, which is 22 bp upstream of the Z-form alternating purine-pyrimidine site. Lastly the essential region constituting the

5   origin of replication in pBR322 extends from the RNA/DNA junction at 2536 to position 2360, 32 base pairs from the weak Z-form site detected with Λ-Co(DIP)$_3{}^{3+}$ (65,93). Thus there appears to be a remarkable correspondence between Z-DNA sites recognized by the cobalt complex and

10   the ends of genetic coding elements. It is tempting to suggest that the Z-DNA conformationn might provide a general structural signal or punction mark which demarcates the ends of these genes. Consistent with this idea, the Z-conformation has been shown to provide a

15   poor template for transcriptional activity with E. coli RNA polymerase (94). This notion is consistent also with the location of alternating purine-pyrimidine tracts in SV40 DNA enhancer sequences which bind anti-Z-DNA antibodies (30), to the d(GT)$_n$ tracts at the ends of

20   yeast chromosomes (95), and to the alternating purine-pyrimidine long terminal repeats in mouse mammary tumor virus (96). Moreover recent experiments with mung bean nuclease in the malaria parasite Plasmodium have demonstrated that a particular conformation rather than a

25   sequence appears to be shared by gene termination sites (97). These correlations of location with conformation are intriguing and support the notion that DNA polymorphism may be involved in gene expression, that specific sequences may adopt specific local conformation which

30   contain information, and that chromosomal regulation may involve DNA conformation-specific signals.

In sum, the results presented herein indicate that several discrete Z-DNA sites exist under native physio-

35   logical conditions in pBR322. The positions of these

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sites mark the ends of genetically distinct coding
elements in the plasmid. A cobalt complex of this
invention, e.g. Λ -Co(DIP)$_3{}^{3+}$ thus provides a photo-
activated site-specific cleaving agent that is useful to
map these sites and should be helpful in establishing a
relationship between the locations of Z-DNA segments
and its biological function.

V. <u>Anti-tumor Activity</u>

Complexes of this invention showed high potency against
leukemia cells in the previously described screen. These
complexes should be useful as anti-tumor agents, and
should be active <u>in vivo</u> as well, e.g. in a composition
containing a pharmaceutically acceptable carrier.

The results set forth in Table 2, above also indicate a
synergistic effect when use of a cobalt complex is
combined with ultraviolet radiation. A decrease in the
amount of cobalt required for cell death of greater than
about 10-fold, and in some cases up to 50-fold has
thereby been observed.

Isolation of chromatin showed extensive DNA cleavage.
Hence it appears that the complexes can pass into the
cell, remain intact therein and interact with DNA as a
cellular target.

Since these complexes contain large, planar ligands,
selective intercalation is optimized. Individual enan-
tiomers and mixed-ligand complexes should also be useful
in this and other embodiments of the invention.
The Λ Z-specific enantiomers should be especially useful
in anti-tumor compositions and uses.

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References (I)

1.   Lerman, L.S. J. Mol. Biol. 1961, 3, 18.

5   2.   Berman, H.M.; Young, P.R. Ann. Rev. Biophys. Bioeng. 1981, 10, 87.

3.   Gale, E.F.; Cundliffe, E.; Reynolds, P.E.; Richmond, M.H.; Waring, M. "The Molecular Basis of
10 Antibiotic Action"; Wiley: London, 1972; p 173.

4.   Waring, M.J. J. Mol. Biol. 1970, 54, 247.

5.   Neidle, S. Prog. Med. Chem. 1979, 16, 151.
15

6.   (a) Espejo, R.T.; Lebowitz, J. Anal. Biochem. 1976, 72, 95. (b) Wang, J.C. J. Mol. Biol. 1974, 89, 783.

7.   Lippard, S.J. Acc. Chem. Res. 1979, 11, 211.
20

8.   Lippard, S.J.; Bond, P.J.; Wu, K.C.; Bauer, W.R., Science (Washington, D.C.) 1976, 194, 726.

9.   (a) Jennette, K.W.; Lippard, S.J.; Vassiliades,
25 G.A.; Bauer, W.R. Proc. Natl. Acad. Sci. U.S.A. 1974, 71, 3839.   (b) Bond, P.J.; Langridge, R.; Jennette, K.W.; Lippard, S.J. Ibid. 1975, 72, 4285. (c) Barton, J.K.; Lippard, S.J. Biochemistry 1979, 18, 2661.   (d) Howe-Grant, M.; Wu, K.C.; Bauer, W.R.; Lippard, S.J.
30 Ibid. 1976, 15, 4339.

10.   Wang, A.H.; Nathans, J.; van der Marel, G.; van Boom, J.H.; Rich, A. Nature (London) 1978, 276, 471.

35

-66-

11. Wong, Y.S.; Lippard, S.J. J. Chem. Soc. Chem. Commun. 1977, 824.

12. (a) Hertzberg, R.P.; Dervan, P.B. J. Am. Chem. Soc. 1982, 104, 313. (b) Van Dyke, M.W.; Hertzberg, R.P.; Dervan, P.B. Proc. Natl. Acad. Sci. U.S.A. 1982, 72, 5470. (c) Van Dyke, M.W.; Dervan, P.B. Biochemistry 1983, 22, 2373.

13. (a) Reich, K.A.; Marshall, L.E.; Graham, D.R.; Sigman, D.S. J. Am. Chem. Soc. 1981, 103, 3582. (b) Marshall, L.E.; Graham, D.R.; Reich, K.A.; Sigman, D.S. Biochemistry 1981, 20, 244. (c) Pope, L.E.; Sigman, D.E. Proc. Natl. Acad. Sci. U.S.A. 1984, 81, 3.

14. Cartwright, I.L.; Elgin, S.C.R. Nucleic. Acids Res. 1982, 10, 5835.

15. Barton, J.K.; Dannenberg, J.J.; Raphael, A.L. J. Am. Chem. Soc. 1982, 104, 4967.

16. Wang, A.H.-J., Quigley, G.J., Kolpak, F.J., Crawford, J.L., van Boom, J.H., van der Marel, G. & Rich, A. (1979) Nature (London) 282, 680-686.

17. Pohl, F.M. & Jovin, T.M. (1972) J. Mol. Biol. 67, 375-396.

18. Zacharias, W.., Larson, J.E., Klysik, J., Stirdivant, S.M. & Wells, R.D. (1982) J. Biol. Chem. 257, 2775-2782.

19. Behe, M. & Felsenfeld, G. (1981) Proc. Natl. Acad. Sci. USA 78, 1619-1623.

20. Santella, R.M., Grunberger, D., Weinstein, E.B. & Rich, A. (1981) Proc. Natl. Acad. Sci. USA 78, 1451-1455.

5   21. Sage, E. & Leng, M. (1980) Proc. Natl. Acad. Sci. USA 77, 4597-4601.

22. Wells, R.D., Miglietta, J.J., Klysik, J., Larson, J.E., Stirdivant, S.M. & Zacharias, W. (1982) J. Biol.
10   Chem. 257, 10166-10171.

23. Ushay, H.M., Santella, R.M., Caradonna, J.P., Grunberger, D. & Lippard, S.J. (1982) Nucleic Acids Res. 10, 3573-3588.

15 ·
24. Haniford, D.B. & Pulleybank, D.E. (1983) Nature (London) 302, 632-634.

25. Nordheim, A. & Rich, A. (1983) Proc. Natl. Acad.
20   Sci. USA 80, 1821-1825.

26. McIntosh, L.P., Greiger, I., Eckstein, F., Zarling, D.A., van de Sande, J.H. & Jovin, T.M. (1983) Nature (London) 304, 83-86.

25
27. Singleton, C.K., Klysik, J., Stirdivant, S.M. & Wells, R.Dp. (1982) Nature (London) 299, 312-316.

28. Klysik, J., Stirdivant, S.M., Larson, J.E., Hart,
30   P.A. & Wells, R.D. (1981) Nature (London) 290, 672-677.

29. Peck, L.J., Nordheim, A., Rich, A. & Wang, J.C. (1982) Proc. Natl. Acad. Sci. USA 79, 4560-4564.

35

-68-

30. Nordheim, A. & Rich, A. (1983) Nature (London) 303, 674-679.

31. Nordheim, A., Pardue, M.L., Lafer, E.M., Moller, A., Stollar, B.D. & Rich, A. (1981) Nature (London) 294, 417-422.

32. Malfoy, B., Rousseau, M. & Leng, M. (1982) Biochemistry 21, 5463-5467.

33. Miller, A., Gabriëls, J.E., Lafer, E.M., Nordheim, A., Rich, A. & Stollar, B.D. (1982) J. Biol. Chem. 257, 12081-12085.

34. Barton, J.K., Danishefsky, A. & Goldberg, J. (1984) J. Am. Chem. Soc., 106, 2172-2176.

35. Barton, J.K. (1983) J. Biomol. Struct. Dyn. 1, 621-632.

36. Bond, P.J., Langridge, R.J., Jennette, K.W. & Lippard, S.J. (1975) Proc. Natl. Acad. Sci. USA 72, 4825-4829.

37. Watts, R.J. & Crosby, G.A. (1971) J. Am. Chem. Soc. 93, 3184-3188.

38. Lin, C.T., Bottcher, W., Chou, M., Creutz, C. & Sutin, N. (1976) J. Am. Chem. Soc. 98, 6536-6544.

39. (a) Hertzberg, R.P.; Dervan,P.B. J. Am. Chem. Soc. 1982, 104, 313. (b) Schultz, P.G.; Taylor, J.S.; Dervan, P.B. Ibid. 1982, 104, 6861. (c) Schultz, P.G.; Dervan, P.B. Proc. Natl. Acad. Sci. U.S.A. 1983, 80, 6834. (d) Taylor, J.S.; Schultz, P.G.; Dervan, P.B. Tetrahedron 1984, 40, 457.

-69-

40. (a) Sigman, D.S.; Graham, D.R.: Marshall, L.E.; Reich, K.A. J. Am. Chem. Soc. 1980, 102, 5419; (b) Biochemistry 1981, 20, 244. (c) Que, B.G.; Downey, K.M.; So, A.G. Ibid. 1980, 19, 5987. (d) Recently a phenan-throline-copper complex was shown to cleave right-handed DNA conformations preferentially. See: Pope, L.A.; Sigman, D.S. Proc. Natl. Acad. Sci. U.S.A. 1984, 81,3.

41. (a) Van Dyke, M.W. Hertzberg, R.P.; Dervan, P.B. Proc. Natl. Acad. Sci. U.S.A. 1982, 79, 5470. (b) Cartwright, I.L.; Hertzberg, R.P.; Dervan, P.B.; Elgin, S.C.R. Proc. Natl. Acad. Sci. U.S.A. 1983, 80, 3213. (c) Cartwright, I.L.; Elgin, S.C.R. Nucleic Acids res. 1982, 10, 5835. (d) Jessee, B.; Gargiulo, G.: Razvi, R.; Woral, A. Ibid. 1982, 10, 5823. (e) Van Dyke, M.W.; Dervan, P.B. Biochemistry 1983, 22, 2372. (f) Lane, M.J.;Dabrowiak, J.C.; Vournakis, J.N. Proc. Natl. Acad. Sci. U.S.A. 1983, 80, 3260.

42. (a) "Bleomycin: Chemical, Biochemical and Biological Aspects"; Hecht, S.M., Ed.; Springer-Verlag: New York, 1979. (b) Sausville, E.A.; Peisach, J.; Horwitz, S.B. biochemistry 1978, 17, 2740. (c) Burger, R.M.; Peisach, J.; Horwitz, S.B. Life Sci. 1981, 28, 715. (d) Dabrowiak, J.C.J. Inorg. Biochem. 1980, 13,317. (e) Lown, J.W.; Sim, S.K. Biochem. Biophys. Res. Commun. 1977, 77, 1150.

43. Cone, R.; Hasan, S.K.; Lown, J.W.; Morgan, A.R. Can J. Biochem. 1976, 54, 219.

44. Cobalt (III) polyamine complexes generally undergo photoreduction reactions. See: (a) Adamson, A.W. Coord. Chem. Rev. 1968, 3, 169. (b) Balzani, V.; Maggi, L.; Manfrin, M.F.; Bolletta, F. Ibid. 1975, 15, 321. (c) Adamson, A.W. Pure Appl. Chem. 1979, 51, 313.

45. (a) Sutin, N.; Cruetz, C. Pure Appl. Chem. 1980, 52, 2717. (b) Balzani, V.; Moggi, L.; Manfrin, M.F.; Bolletta, F.; Laurence, G.S. Coord. Chem. Rev. 1975, 15, 321.

46. (a) Hager, G.D.; Crosby, G.A.; J. Am. Chem. Soc. 1975, 97, 7031. (b) Hager, G.D.; Watts, R.J. Crosby, G.A. Ibid. 1975, 97, 7037. (c) Elfring, W.H., Jr.; Crosby, G.A. Ibid. 1981, 103, 2683.

47. Circular dichroic spectra of $Zn(phen)_3^{2+}$, optically enriched by dialysis against DNA (15), show that, while the complex does not racemize instantaneously in aqueous solution, loss of optical activity does occur over a period of days. (Barton, J.K.; Dannenberg, J.J.; Raphael, A.L., to be submitted for publication.) Enantiomers have not however been isolated in pure form. See also: Pfeiffer, P.; Nakatsuka, Y. Ber. Dtsch. Chem. Ges. B 1933, 66B, 415.

48. Brandt, W.W.; Dwyer, F.P.; Gyarfas, E.C. Chem. Rev. 1954, 54, 959 and references therein.

49. Gillard, R.D.; Hill, R.E.E. J. Chem. Soc., Dalton Trans. 1974, 1217.

50. McCaffery, A.J.; Mason, S.F.; Norman, B.J. J. Chem. Soc. A 1969, 1428.

51. Yamagishi, A. J. Chem. Soc., Chem. Commun. 1983, 572.

52. J.K. barton, L.A. Basile, and A. Danishefsky, proc. Natl. Acad. Sci. USA, 1984, 81, 1961-1965.

53. Cagle, F.W. Jr., Acta Cryst. 1 158(148).

54. Pohl, F., Jovin, T., Baehr, W. & Holbrook, J. (1972) Proc. Natl. Acad. Sci. USA 69, 3805-3809.

55. van de Sande, J. & Jovin, T. (1982) EMBO J. 1, 115-120.

56. Mirau, P.A. and Kearns, D.R. Nuc. Acids Res. 11, 1931 (1983).

57. Miller, O.J., Miller, D., and Barton, J.K. to be submitted.

58. Dolimore, L.S.; Gillard, R.D. J. Chem. Soc., Dalton Trans. 1973, 933.

59. The low spin d^6 octahedral cobalt(III) complexes are kinetically inert to racemization. See: (a) Ellis, P.; Wilkins, R.G.; Williams, M.J.G.J. Chem. Soc. Chem. Commun. 1957, 4456. (b) Yamamoto, M.; Uwate, Y.; Yamamoto, Y. Inorg. Nucl. Chem. Lett.

60. (a) Rich, A., Nordheim, A., and Want, A. H-J. Ann. Rev. Biochem. 53, 791-846 (1984). (b) Dickerson, R.E., Drew, H.R., Conner, B.N., Wing, R.M., Fratini, A.V. and Kopka, M.L. Science 216, 475-485 (1982) and references therein.

61. (a) Patel, D.J., Kozlowski, S.A., Nordheim, A. and Rich, A. Proc. Natl. Acad. Sci. USA 79, 1413-1417 (1982). (b) Pohl, F.M. and Jovin, T.M. J. Mol. Biol. 67, 375-396 (1972). (c) McIntosh, L.P., Grieger, I., Eckstein, F., Zarling, D.A., van de Sande, J.H., and Jovin, T.M. Nature 304, 83-86 (1983). (d) Haniford, D.B. and Pulleybank, E.D. Nature 302,632-634 (1983).

62. (a) Lilley, D.M.J. Proc. Natl. Acad. Sci. USA 77, 64468-6472 (1980). (b) Saragosti, S., Cereghini, S. and Yaniv, M. J. Mol. Biol 160, 133-146 (1982). (c) Larsen, A. and Weintraub, H. Cell 29, 609-622 (1982).

63. Barton, J.K., Basile, L.A., Danishefsky, A. and Alexandrescu, A. Proc. Natl. Acad. Sci. USA 81, 1961-1965 (1984).

64. Barton, J.K. and Raphael, A.L. J. Am. Chem. Soc. 106, 2466-2468 (1984).

65. (a) Sutcliffe, J.G. Cold Spring Harbor Symp. Quant. Biol. 43, 77-90 (1979). (b) Sutcliffe, J.G. Proc. Natl. Acad. Sci. USA 75, 3737-3741 (1978).

66. Barton, J.K. & Lippard, S.J. (1979) Biochemistry 12, 2661-2668.

67. Reichman, M.E., Rice, S.A., Thomas, C.A. & Doty, P.J. (1954) J. Am. Chem. Soc. 76, 3047-3053.

68. Wells, R.D., Larson, J.E., Grant, R.C., Shortle, B.E. & Cantor, C.R. (1970) J. Mol. Biol. 54, 465-497.

69. Fisher, G.A. (1958) Ann. N.Y. Acad. Sci., 76,673.

70. LePecq, J.B.; Paoletti, C. (1967) J. Mol. Biol. 27,87.

71. Scatchard, G., Ann. N.Y. Acad. Sci., 1949, 51, 660.

72. McGhee, J.D.; von Hippel, P.H., J. Mol. Biol., 1974, 86, 469.

73. Howe-Grant, M.; Lippard, S.J., Biochem., 1979, 18, 5762.

74. Crothers, D.M., Biopolymers, 1968, 6, 575.

75. Pohl, F., Jovin, T., Baehr, W. & Holbrook, J. (1972) Proc. Natl. Acad. Sci. USA 69, 3805-3809.

76. van de Sande, J. and Jovin, T. (1982) EMBO J. 1, 115-120.

77. Wang. A.H.J., Fujii, S., van Boom, J.H. and Rich, A. (1982) Proc. Natl. Acad. Sci. USA 79, 3968-3972.

78. (a) Tullius, T.D. et al., ACS Symp. Ser., 1983, No. 209,51. (b) Cohen et al., Science, 1979, 203, 1014.

79. Peck, L.J.; Wang, J.C. Ibid. 1983, 80, 6206. We thank Professor C. Cantor for a gift of this plasmid.

80. (a) Azorin, F.; Nordheim, A.; Rich, A. EMBO J. 1983, 2, 649. (b) Nordheim, A.; Lafer, E.M.; Peck, L.J.; Wang, J.C.; Stollar, B.D.; Rich, A. Cell 1982, 31, 309. (c) Rich, A.; Nordheim, A.; Azorin, F.J. Biomol. Struct. Dyn. 1983, 1,1.

-74-

81. Stirdivant, S.M., Klysik, J. and Wells, R.D. *J. Biol. Chem.* 257, 10159-10165 (1982).

82. Hauenstein, B.L., Jr.; Dressick, W.J.; Buell, S.L.; Demas, J.N.; DeGraff, B.A. J. Am. Chem. Soc. 1983, 105, 4251.

83. Dwyer, F.P. et al., Nature, 1952, 170, 190.

84. Watts, R.J. and Crosby, G.A. (1971) J. Am. Chem. Soc. 93, 3184-3188.

85. Lin, C.T., Bottcher, W., Chou, M., Creutz, C. and Sutin, N. (1976) J. Am. Chem. Soc. 98, 6536-6544.

86. Cagle, F.W., Jr. (1948) Acta Crystallogr. 1, 158-159.

87. Fiel, R.J., Howard, J.C., Mark, E.H. & Gupta, N.D. (1979) Nucleic Acids. Res. 6, 3093-3118.

88. Pasternack, R.F., Gibbs, E.J. & Villafranca, J.J. (1982) Biochemistry 22, 2406-2414.

89. Arnott, S., Chandrasekaran, R., Birdsall, D.L., Leslie, A.G.W. & Ratliff, R.L. (1980) Nature (London) 283, 743-745.

90. Gupta, G., Bansal, M. & Sasisekharan, V. (1980) Proc. Natl. Acad. Sci. USA 77, 6486-6490.

91. Backman, K. and Boyer, H.W. *Gene* 26, 197-203 (1983).

-75-

92. Brosius, J., Cate, R.L., and Permutter, A.P. J. Biol. Chem. 257, 9205-9210 (1982).

93. Tomizawa, J.I., Ohmori, H. and Bird, R.E. Proc. Natl. Acad. Sci. USA 74, 1865-1869 (1977).

94. Butzow, J.J., Shin, Y.A., and Eichhorn, G.L. Biochemistry 23, 4837-4843 (1984).

95. Walmsley, R.M., Szostak, J.W. Petes, T.D. Nature 302, 84-86 (1983).

96. See, for example Kennedy, N., Knedlitschek, G., Groner, B., Hynes, N.E., Herrlich, P., Michalides, R., and van Ooyen, A.J.J. Nature 295, 622-624 (1982).

97. McCutchan, T.F., Hansen, J.L., Dane, J.B. and Mullins, J.A. Science 225, 625-628 (1984).

98. Dwyer, F.P.; Reid, I.K.; Shulman, A.; Laycock, G.M. and Dixon, S., Aust. J. Exp. Biol. Med. Sci., 1969, 47, 203; Dwyer, F.P.; Mayhew, E.; Roe, E.M.F.; and Shulman, A., Brit. J. Cancer, 1965, 19, 195; Shulman, A. and White, D.O., Chem. Biol. Inter., 1973, 6, 407; Shulman, A. and Laycock, G.M., Chem. Biol. Inter., 1977, 16, 89.

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<u>What is claimed is</u>:

1.   A coordination complex having the formula $(R)_3$---Co(III), wherein R comprises 1,10-phenanthroline or a substituted derivative thereof and R is bound to Co by a coordination bond or salt thereof.

2.   A complex of claim 1, wherein the substituted derivative of 1,10-phenanthroline comprises 4,7-diamino-1,10-phenanthroline;   3,8-diamino-1,10-phenanthroline; 4,7-diethylenediamine-1,10-phenanthroline;   3,8-diethylenediamine-1,10-phenanthroline;   4,7-dihydroxyl-1,10-phenanthroline; 3,8-dihydroxyl-1,10-phenanthroline; 4,7-dinitro-1,10-phenanthroline;   3,8-dinitro-1,10-phenanthroline;   4,7-diphenyl-1,10-phenanthroline;   3,8-diphenyl-1,10-phenanthroline;   4,7-dispermine-1,10-phenanthroline, or 3,8-dispermine-1,10-phenanthroline.

3.   The optically resolved Δ isomer of a complex of claim 1

4.   The optically resolved Λ isomer of a complex of claim 1.

5.   The optically resolved Δ isomer of a complex of claim 2.

6.   The optically resolved Λ isomer of a complex of claim 2.

7.   A method for labeling DNA with a complex of the formula $(R)_3$---M, wherein R comprises 1,10-phenanthroline or a substituted derivative thereof, M comprises a suitable transition metal, and R is bonded to M by a coordination bond, which comprises contacting the DNA

with the complex under suitable conditions such that the complex binds to the DNA by intercalation.

8.   A method of claim 7, wherein M is ruthenium(II).

9.   A method of claim 7, wherein M is cobalt (III).

10.   A method of claim 7, wherein the substituted derivative of 1,10-phenanthroline comprises 4,7-diamino-1,10-phenanthroline;   3,8-diamino-1,10-phenanthroline;   4,7-diethylenediamine-1,10-phenanthroline;   3,8-diethylene-diamine-1,10-phenanthroline; 4,7-dihydroxyl-1,10-phenan-throline;   3,8-dihydroxyl-1,10-phenanthroline;   4,7-dini-tro-1,10-phenanthroline;   3,8-dinitro-1,10-phenanthro-line;   4,7-diphenyl-1,10-phenanthroline;   3,8-diphenyl-1,10-phenanthroline; 4,7-dispermine-1,10-phenanthroline, or 3,8-dispermine-1,10-phenanthroline.

11.   A DNA molecule labeled with a complex of the formula $(R)_3$ ---M, wherein R is 1,10-phenanthroline or a substituted derivative thereof; M is a suitable transi-tion metal; R is bonded to M by a coordination bond and the complex is bound to the DNA by intercalation.

12.   A labeled DNA molecule of claim 11, wherein M is ruthenium(II).

13.   A labeled DNA molecule of claim 11, wherein M is cobalt(III). ●

14.   A labeled DNA molecule produced by the method of claim 7.

15.   A method for selectively labeling Z-DNA with the lambda enantiomer of a complex of the formula $(R)_3$ ---M,

wherein R comprises 1,10-phenanthroline or a substituted
derivative thereof, M comprises a suitable transition
metal, and R is bonded to M by a coordination bond,
which comprises contacting the DNA with the lambda
5 enantiomer of the complex under suitable conditions such
that the complex binds to the Z-DNA.

16. A method of claim 15, wherein M is ruthenium(II).

10 17. A method of claim 15, wherein M is cobalt(III).

18. A method of claim 15, wherein the substituted
derivative of 1,10-phenanthroline comprises 4,7-diamino-
1,10-phenanthroline; 3,8-diamino-1,10-phenanthroline;
15 4,7-diethylenediamine-1,10-phenanthroline; 3,8-diethy-
lenediamine-1,10-phenanthroline; 4,7-dihydroxyl-1,10-
phenanthroline; 3,8-dihydroxyl-1,10-phenanthroline; 4,7-
dinitro-1,10-phenanthroline; 3,8-dinitro-1,10-phenan-
throline; 4,7-diphenyl-1,10-phenanthroline; 3,8-diphe-
20 nyl-1,10-phenanthroline; 4,7-dispermine-1,10-phenanthro-
line, or 3,8-dispermine-1,10-phenanthroline.

19. A labeled DNA molecule containing Z-DNA and a
lambda enantiomer of a complex of the formula $(R)_3$---M,
25 wherein R is 1,10-phenanthroline or a substituted deriv-
ative thereof, M is a suitable transition metal and R is
bound to M by a coordination bond, the complex being
bound to the Z-DNA.

30 20. A labeled DNA molecule of claim 19, wherein M is
ruthenium (II).

21. A labeled DNA molecule of claim 19, wherein M is
cobalt(III).
35

22. A labeled DNA molecule of claim 19, wherein the substituted derivative of 1,10-phenanthroline comprises 4,7-diamino-1,10-phenanthroline; 3,8-diamino-1,10-phenanthroline; 4,7-diethylenediamine-1,10-phenanthroline; 3,8-diethylenediamine-1,10-phenanthroline; 4,7-dihydroxyl-1,10-phenanthroline; 3,8-dihydroxyl-1,10-phenanthroline; 4,7-dinitro-1,10-phenanthroline; 3,8-dinitro-1,10-phenanthroline; 4,7-diphenyl-1,10-phenanthroline; 3,8-diphenyl-1,10-phenanthroline; 4,7-dispermine-1,10-phenanthroline, or 3,8-dispermine-1,10-phenanthroline.

23. A labeled DNA molecule produced by the method of claim 15.

24. A method for detecting the presence of Z-DNA which comprises selectively labeling Z-DNA according to claim 15 and detecting the presence of the complex bound to Z-DNA.

25. A method for nicking double-stranded DNA by effecting breakage of at least one phosphodiester bond along the DNA which comprises contacting the DNA with a complex of claim 1 under suitable conditions such that the complex binds to the DNA to form an adduct and irradiating the adduct so formed with a sufficient dose of ultraviolet radiation of an appropriate wavelength so as to nick the DNA at the site(s) of intercalation.

26. A method for cleaving double-stranded DNA which comprises nicking the DNA according to claim 25 and treating the nicked DNA so produced with a suitable enzyme capable of cleaving single-stranded DNA under effective conditions to cleave the nicked DNA at the site of the nick(s).

27. A method for selectively nicking Z-DNA by effecting breakage of at least one phosphodiester bond along the Z-DNA which comprises contacting a DNA molecule containing a Z-DNA sequence with a complex of claim 4 under

5 suitable conditions such that the complex binds to the Z-DNA to form an adduct and irradiating the adduct so formed with a sufficient dose of ultraviolet radiation of an appropriate wavelength so as to nick the DNA at the site(s) of intercalation.

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28. A method for selectively cleaving double-stranded Z-DNA which comprises selectively nicking the Z-DNA according to claim 27 and treating the nicked DNA so produced with a suitable enzyme capable of cleaving

15 single-stranded DNA under effective conditions to cleave the nicked DNA at the site of the nick(s).

29. A method for killing a portion of population of appropriate tumor cells which comprises contacting the

20 tumor cells under suitable conditions with an effective amount of the lambda enantiomer of a coordination complex of the formula $(R)_3$---M, wherein R comprises 1,10-phenanthroline or a substituted derivative thereof, M comprises a suitable transition metal, and R is bonded

25 to M by a coordination bond, to kill the tumor cells.

30. A method of claim 29, wherein M is ruthenium(II).

31. A method of claim 29, wherein M is cobalt(III).

30

32. A complex of claim 29, wherein the substituted derivative of 1,10-phenanthroline comprises 4,7-diamino-1,10-phenanthroline; 3,8-diamino-1,10-phenanthroline; 4,7-diethylenediamine-1,10-phenanthroline; 3,8-diethy-

35 lenediamine-1,10-phenanthroline; 4,7-dihydroxyl-1,10-

-81-

phenanthroline; 3,8-dihydroxyl-1,10-phenanthroline; 4,7-dinitro-1,10-phenanthroline;　3,8-dinitro-1,10-phenan-throline;　4,7-diphenyl-1,10-phenanthroline;　3,8-di-phenyl-1,10-phenanthroline;　4,7-dispermine-1,10-phenan-

5　throline, or 3,8-dispermine-1,10-phenanthroline.

33.　A method of claim 29, wherein the tumor cells are present in a subject and the contacting is effected by administering the coordination complex to the subject.

10

34.　A method for killing a portion of a population of appropriate tumor cells which comprises contacting the tumor cells under suitable conditions with an effetive amount of a coordination complex of claim 1 to kill the

15　tumor cells.

35.　A method of claim 34 which further comprises irra-diating the tumor cells with a suitable dose of ultravi-olet radiation of an appropriate wavelength at a suita-

20　ble time after the tumor cells have been contacted with the complex, permitting the complex to nick DNA.

36.　A pharmaceutical composition for the treatment of tumor cells in a subject which comprises an effective

25　anti-tumor amount of the lambda enantiomer of a complex of the formula $(R)_3$---M, wherein R comprises 1,10-phe-nanthroline or a substituted derivative thereof, M comprises a suitable transition metal, and R is bonded to M by coordination bonds or a salt thereof, and a

30　pharmaceutically acceptable carrier.

37.　A pharmaceutical composition of claim 36, wherein M is ruthenium(II).

35

38. A pharmaceutical composition of claim 36, wherein M is cobalt(III).

39. A pharmaceutical composition of claim 36, wherein the substituted derivative of 1,10-phenanthroline comprises 4,7-diamino-1,10-phenanthroline; 3,8-diamino-1,10-phenanthroline; 4,7-diethylenediamine-1,10-phenanthroline; 3,8-diethylenediamine-1,10-phenanthroline; 4,7-dihydroxyl-1,10-phenanthroline; 3,8-dihydroxyl-1,10-phenanthroline; 4,7-dinitro-1,10-phenanthroline; 3,8-dinitro-1,10-phenanthroline; 4,7-diphenyl-1,10-phenanthroline; 3,8-diphenyl-1,10-phenanthroline; 4,7-dispermine-1,10-phenanthroline, or 3,8-dispermine-1,10-phenanthroline.

40. A pharmaceutical composition for the treatment of tumor cells in a subject which comprises an effective anti-tumor amount of a complex of claim 1 and a pharmaceutically acceptable carrier.

41. A pharmaceutical composition for the treatment of tumor cells in a subject which comprises an effective anti-tumor amount of a complex of claim 2 and a pharmaceutically acceptable carrier.

42. A method for treating a subject afflicted with tumor cells so as to cause regression of the tumor cells which comprises administering to the subject by a suitable route a composition of claim 36 in an amount sufficient to cause regression of the tumor cells.

43. A method for treating a subject afflicted with tumor cells so as to cause regression of the tumor cells which comprises administering to the subject by a suitable route a composition of claim 37 in an amount sufficient to cause regression of the tumor cells.

44. A method for treating a subject afflicted with tumor cells so as to cause regression of the tumor cells which comprises administering to the subject by a suitable route a composition of claim 38 in an amount suffi-
5   cient to cause regression of the tumor cells.

45. A method for treating a subject afflicted with tumor cells so as to cause regression of the tumor cells which comprises administering to the subject by a suitable
10   ble route a composition of claim 39 in an amount suffi-cient to cause regression of the tumor cells.

46. A method for treating a subject afflicted with tumor cells so as to cause regression of the tumor cells
15   which comprises administering to the subject by a suitable route a composition of claim 40 in an amount suffi-cient to cause regression of the tumor cells.

47. A method for treating a subject afflicted with
20   tumor cells so as to cause regression of the tumor cells which comprises administering to the subject by a suitable route a composition of claim 41 in an amount suffi-cient to cause regression of the tumor cells.

25   48. A method of claim 42, wherein the route of admini-stration is parenteral.

49. A method of claim 42, wherein the route of admini-stration is topical.
30

50. A method of claim 46, wherein the route of admini-stration is parenteral.

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51.   A method of claim 46, wherein the route of administration is topical.

52.   A method of claim 44 which further comprises irradiating the tumor cells with a suitable dose of ultraviolet radiation of an appropriate wavelength permitting the complex to nick DNA at a suitable time after the administration of the composition to the subject.

53.   A method of claim 46 which further comprises irradiating the tumor cells with a suitable dose of ultraviolet radiation of an appropriate wavelength permitting the complex to nick DNA at a suitable time after the administration of the composition to the subject.

FIGURE 1

PHASE DROSOPHILA

RuDIP
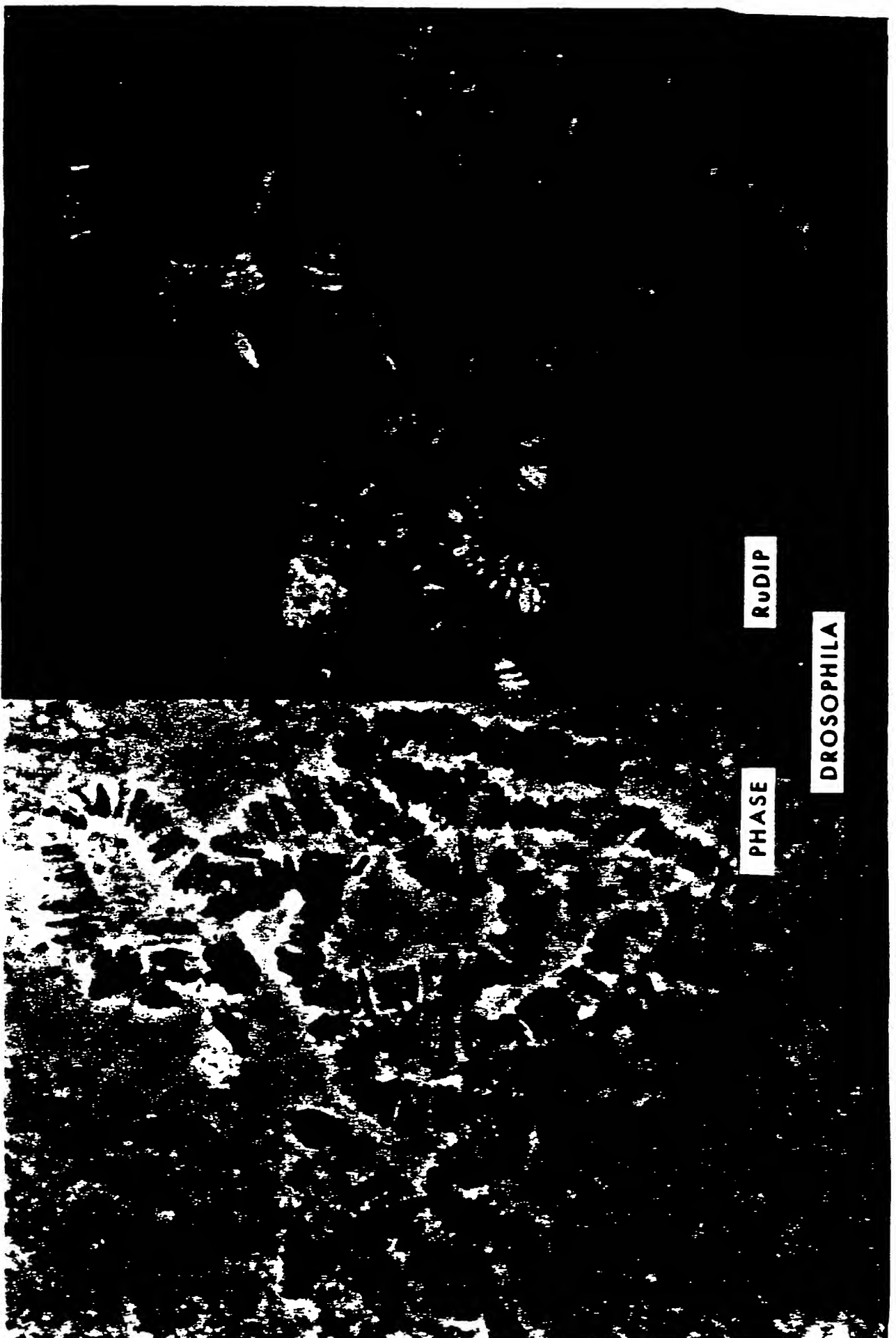
2/12

Fig. 2

Fig. 3

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Fig. 4
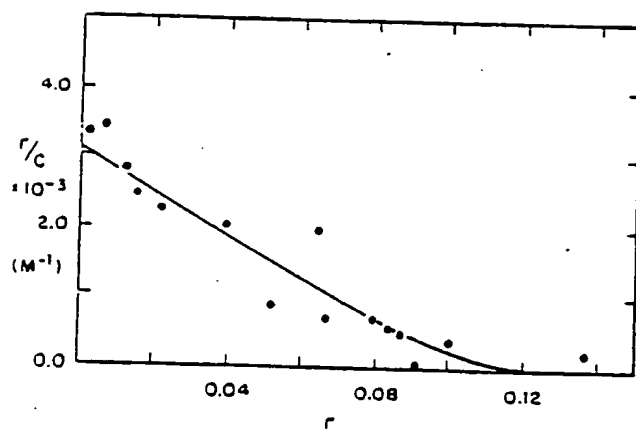
Fig. 5
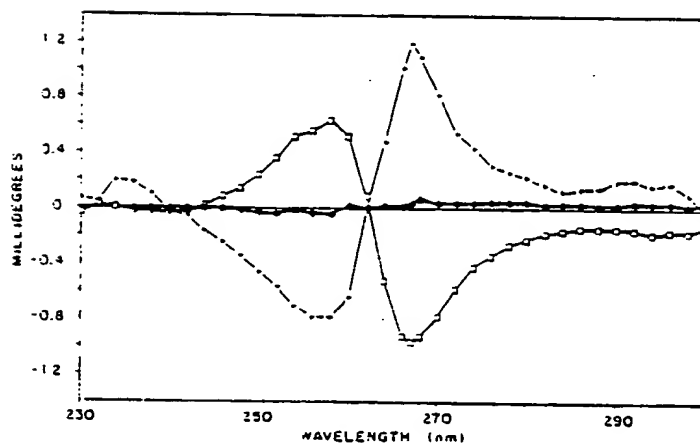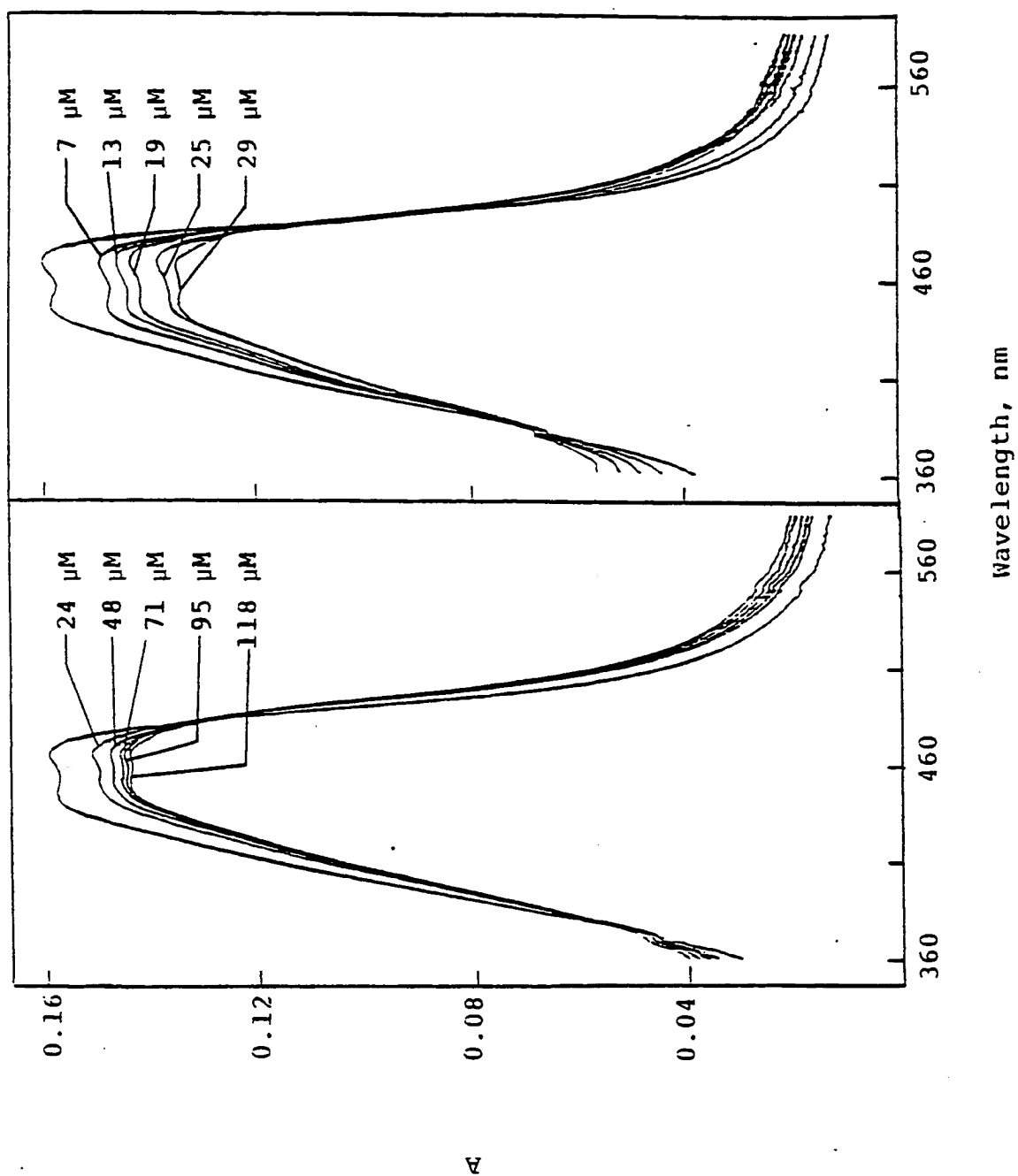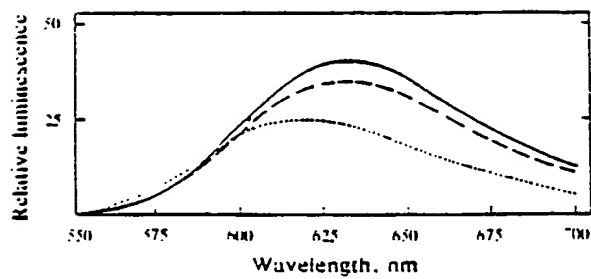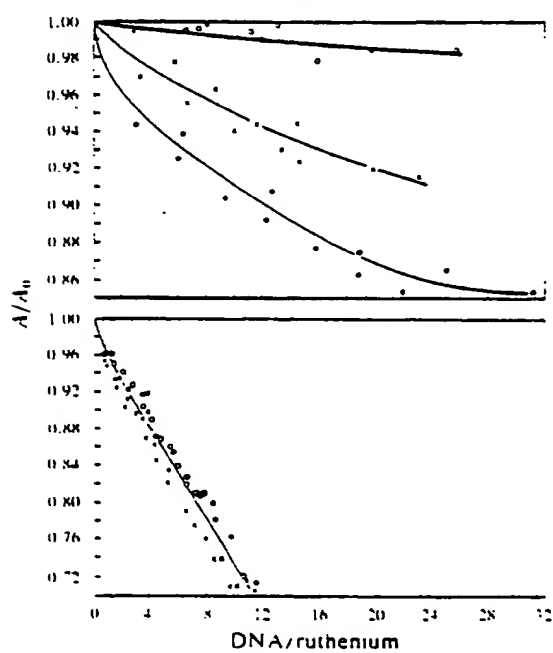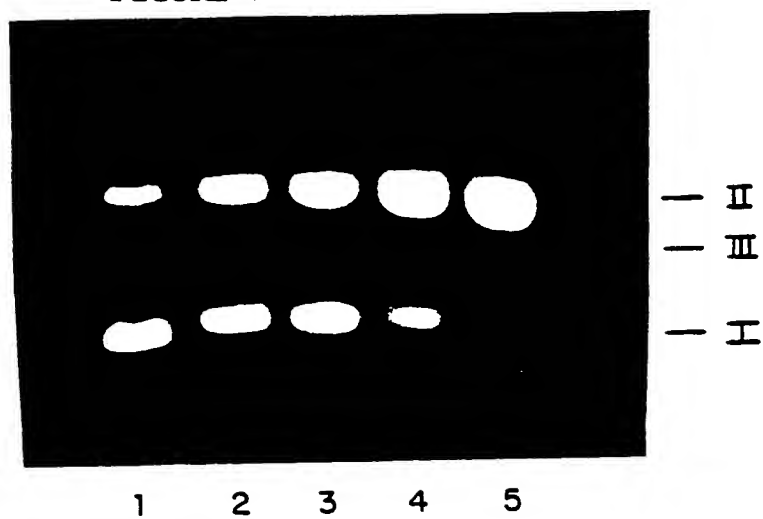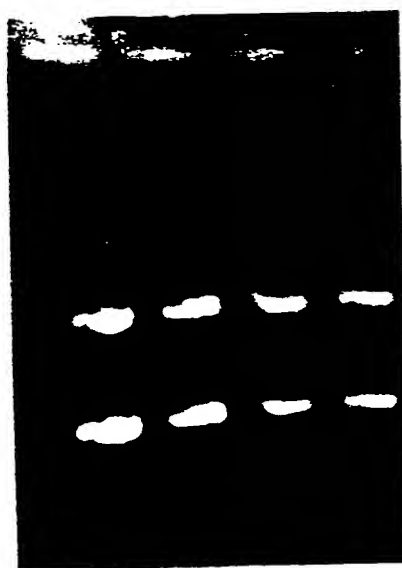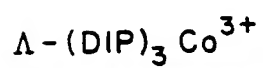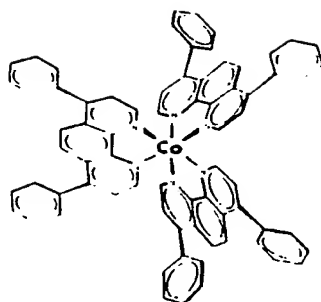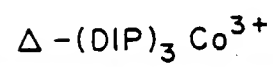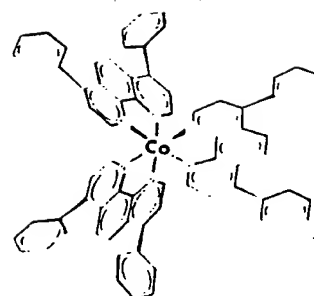
Fig. 6

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Fig. 7

Fig. 8

FIGURE 9

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Fig. 10
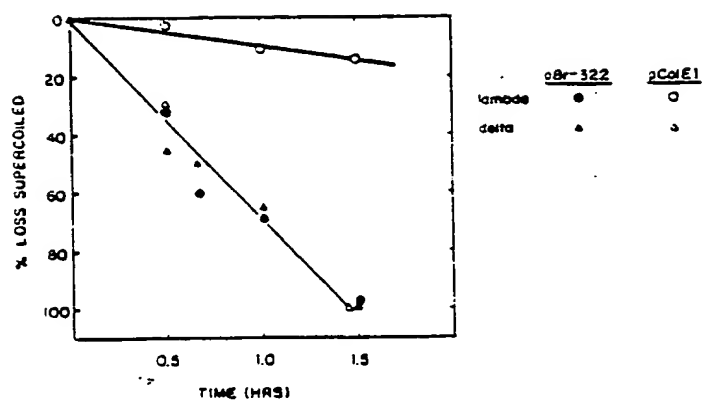
Fig. 11
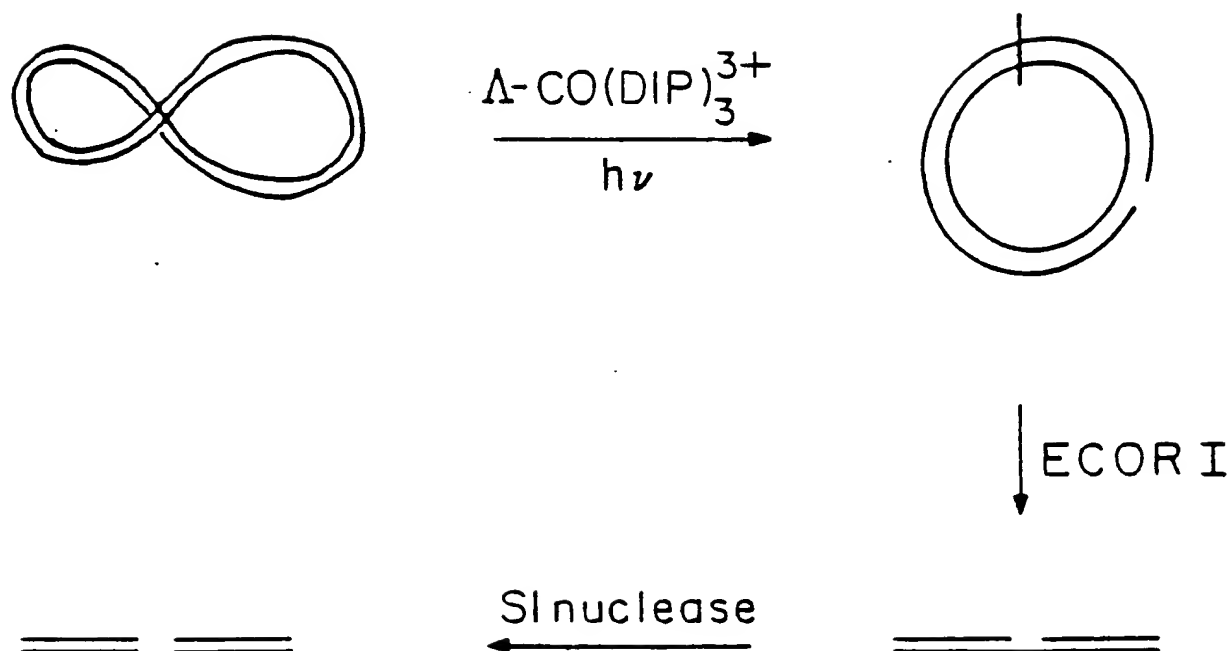
Fig. 12
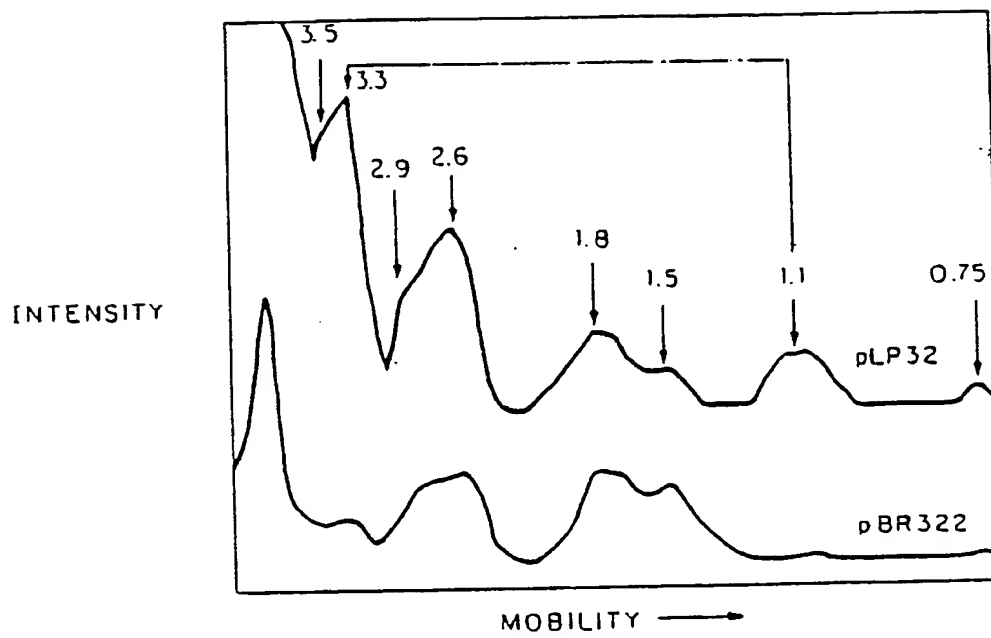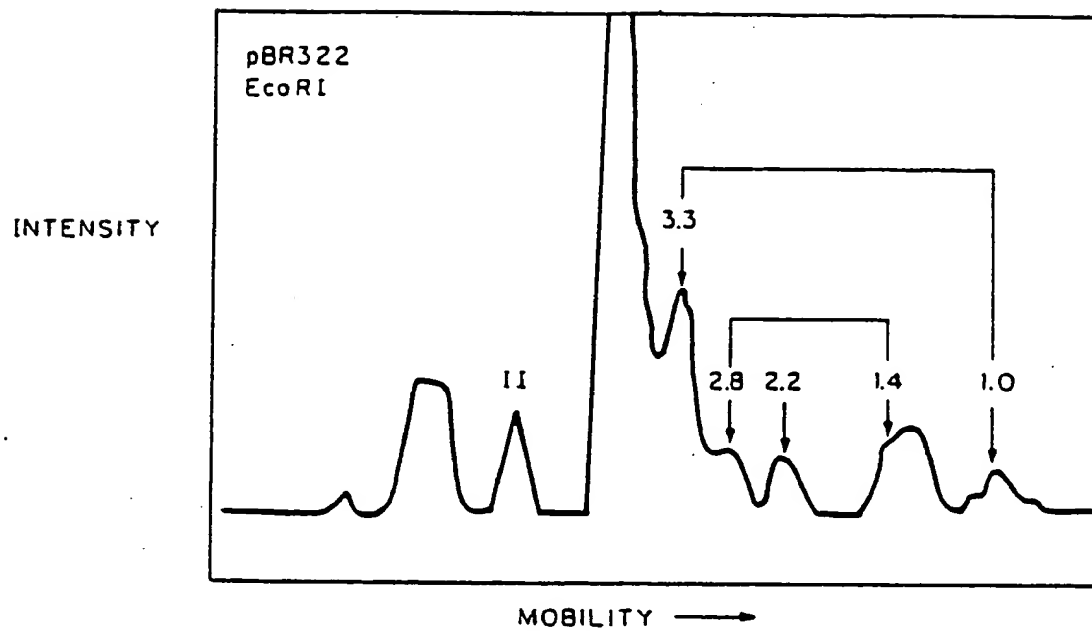
Fig. 13A

Fig. 13B
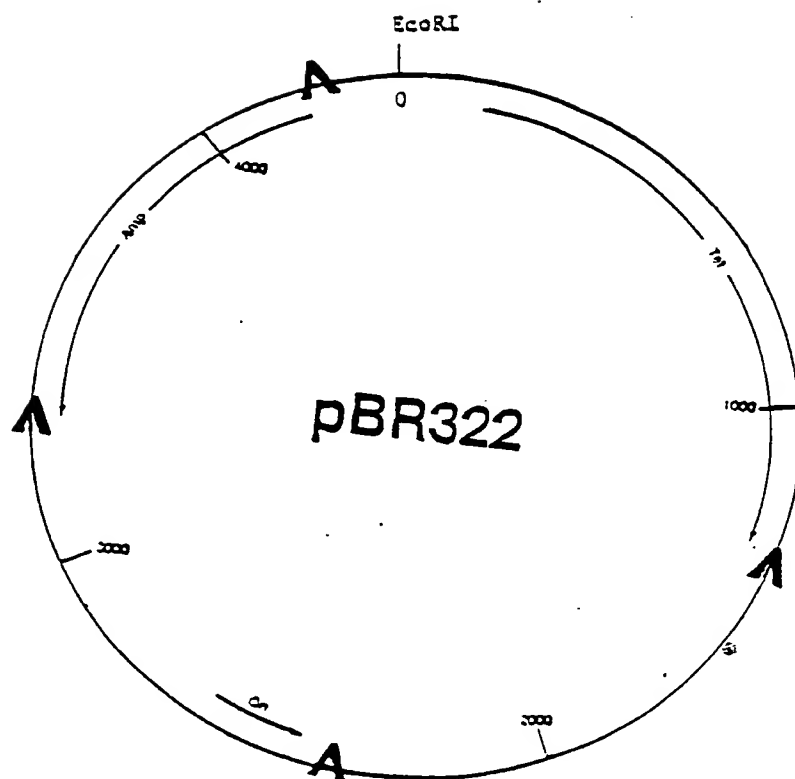
Fig. 14
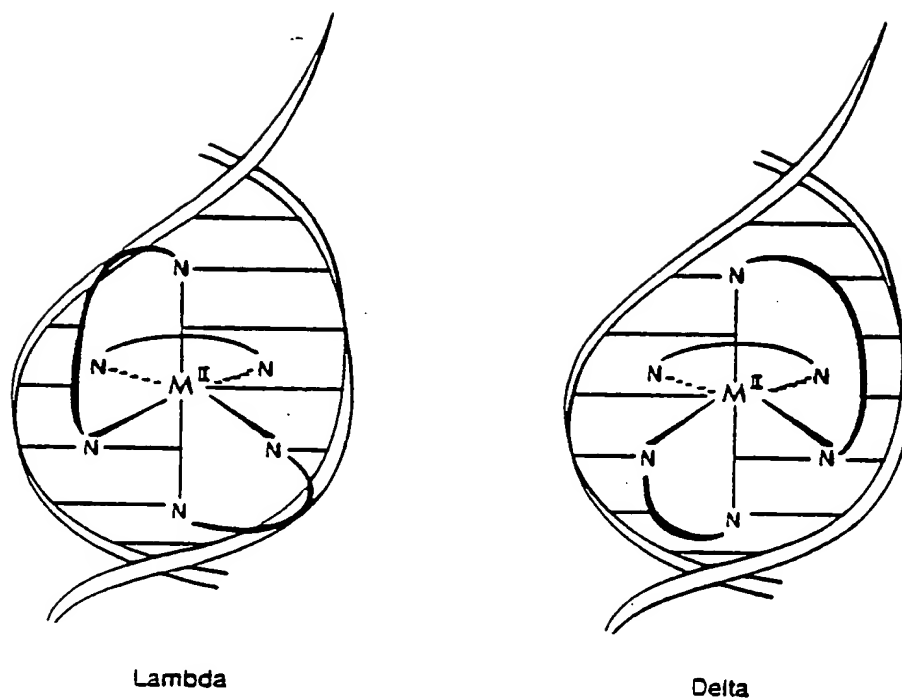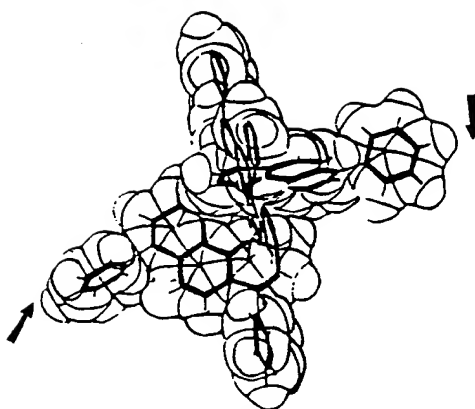
Lambda   Delta
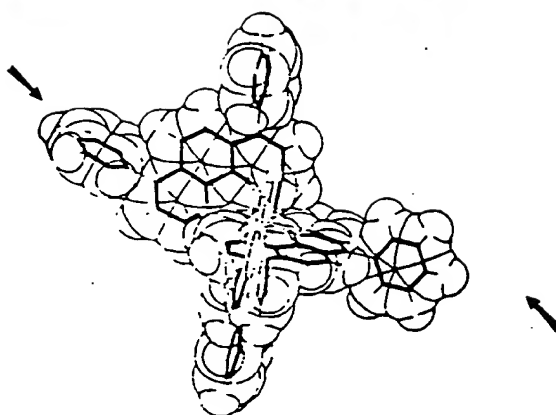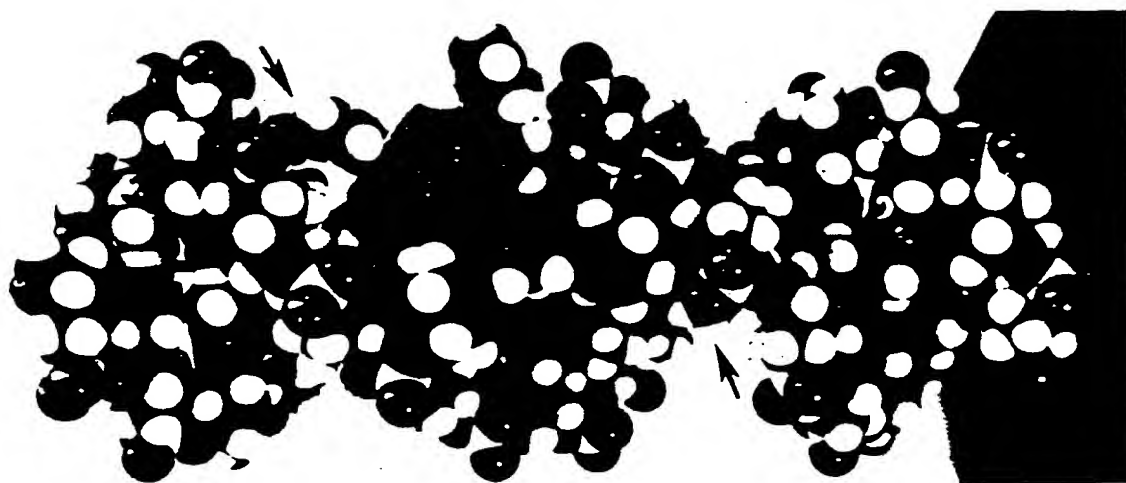
FIGURE 15

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

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U.S. 424/9, 131; 556/136, 138; 546/88; 536/22; 435/6
INT. Cl.4   A61K 49/00;A01N 59/16;C07D 471/04;C07H 19/00; C12Q 1/68
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II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/1.1, 9, 131, 149; 546/10,88; 556/136, 138; 536/22 435/6; 935/77, 78

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER SEARCH: CHEMICAL ABSTRACTS 1969-1986 Biosis 1969-1986

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	N, Proc. Natl. Acad. Sci. USA, Vol. 81, issued April 1984, J. K, Barton et al 'Chiral probes for... tris (4,7-diphenylphenanthroline)ruthenium (II)', pages 1961-1965	1-8,10-12, 14-16, 18-20,22-24
Y,P	N, J. Amer. Chem. Soc., Vol. 107, issued February 1985, J. K. Barton et al, 'Chiral discrimination...to B-DNA', pages 708-709	1-24,29-53
Y	N, J. Amer. Chem. Soc., Vol. 104, No. 18, issued September 1982, J. K. Barton et al, 'Enantiomeric Selectivity...to DNA', pages 4967-4969.	1-24
Y	N, J. Biol. Chem., Vol. 257, No. 20, issued October 1982, L. M. Pope et al. 'Products of DNA...Complex', pages 12121-12128	1,2,25, 26

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
04 April 1986	0 8 APR 1986
International Searching Authority [1]	Signature of Authorized Officer [10]
ISA/US	Jeremy M. Jay

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
Y	N, Biochemistry, Vol. 23, No. 14, issued December 1984, Y. Pommier et al, 'Formation and rejoining...intercalating agents', pages 3194-3201	1,25,26, 34,35
X	N, J. Amer. Chem. Soc., Vol. 106, No. 7, issued April 1984, J. K. Barton et al, 'Tris(phenanthroline)ruthenium (II): ...DNA', pages 2172-2176	1-8,10-12,14-16, 18-20,22-28

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers............ because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers............, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	N, J. Amer. Chem. Soc., Vol. 106, No. 8, issued April 1984, J. K. Barton et al, 'Photoactivated stereospecific... Cobalt (III) Complexes', pages 2466-2468	1-28
Y	N, Inorganic Chem., Vol. 16, No. 4, issued March 1977, M. J. Clarke, 'Linkage isomers of Pentaammineruthenium-Hypoxanthine complexes', pages 738-744.	1,7,11, 15,19,29, 34,36,40, 46
X	N, Chemical Abstracts, Volume 99, no. 17, issued 1983, Akihiko Yamagishi, 'Evidence for stereospecific binding of tris(1,10-phenanthroline)ruthenium(II) to DNA is provided by electronic dichroism" see page 244, column 2, the abstract no. 135693t, J. Chem. Soc. Chem. Commun. 1983, 10, 572-573 (Eng.)	1-6